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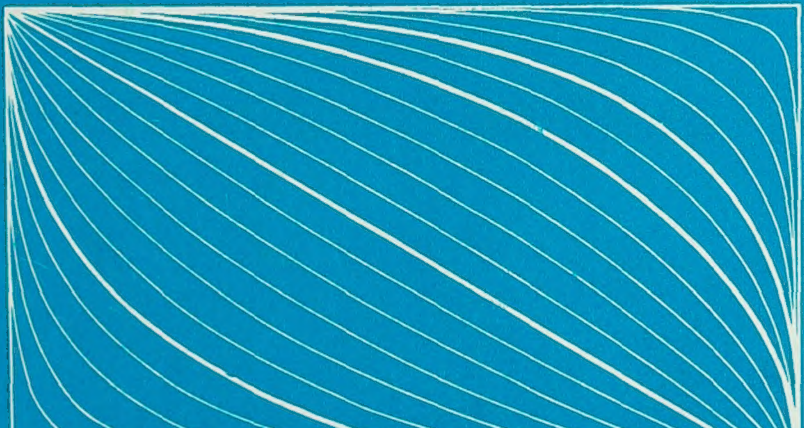
J. Gielis, T. Gerats
(eds.)

Aspects of morphogenesis of leaves, flowers and somatic embryos



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J. Gielis & T. Gerats
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Aspects of morphogenesis of leaves, flowers and somatic embryos

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Foreword

Plant morphogenesis and plant morphology are among the most intruiging fields of botanical science. Nowadays physiological, genetic and molecular approaches are used in combination to unravel the underlying forces that direct "genetic codes" into coherent and fascinating forms, capable of growing in a range of environments. Enormous progress has been made in recent years in understanding plant morphogenesis and developmental processes.

Besides the integration of classical fields, new approaches are being opened: ecological and biophysical mechanisms are increasingly being recognised as playing crucial roles in the transduction of message encoded in the genome. Bio-mathematical aspects can also aid in understanding plant development.

Tissue culture techniques are very important in plant production in both horticulture and agriculture and they continue to be important tools in plant research. While many species can be cultured in vitro, one cannot but continue to wonder how plants, some larger than 50 m in nature, can be grown in small test-tubes, easily adapting to environments so different from their natural habitat.

In this joint meeting an overview of different approaches for the analysis of the development of both vegetative and sporogenic tissues will be presented. We venture to hope that such an overview will be useful for a large audience, including researchers and students, but also a more general public interested in plant morphology and development.

We thank all speakers for their efforts to produce written contributions, ahead of the meeting. Many thanks are due to Professor J. Rammeloo, Director of the National Botanic Garden, and Professor E. Robbrecht, for publishing the papers in *Scripta Botanica Belgica* series. We hope you'll all enjoy the meeting.

Johan Gielis	Belgian Plant and Tissue Culture Group BPTCG
Tom Gerats	FWO Research Network Plant Morphogenesis

Mathematical aspects of real plant modelling

Freddy Van Oystaeyen[1,*], Johan Gielis[2], Reinhart Ceulemans[3]

[1,*] Departement of Mathematics and Computer Science, University of Antwerp, UIA, B-2610 Wilrijk, Belgium; [2] OPRINS PLANT, Sint-Lenaartsesteenweg 91, B-2310 Rijkevorsel, Belgium; [3] Departement of Biology, University of Antwerp, UIA, B-2610 Wilrijk, Belgium

0. Introduction

The shape of an object is its geometric nature. Inevitably any description of the shape of an object and its deformation in time will have to make use of suitable mathematical techniques related to an algebraic, discrete or computational approach to geometry. If we are interested in triangles moving in planes this is very easy, but since we are interested in plant morphogenesis this is a very complex and maybe impossible task. As usual, the impossible is made possible by being satisfied with good enough approximations.

When a geometer "old style" could benefit a lot from being able to "see" 3-dimensional pictures, nowadays we may call upon computer graphics in order to visualise mathematical models of biological structures and processes in a variety of ways, from schematic to photo-realistic. In this paper morphogenesis is used in the sense of "genesis of form" and can be studied at all levels, including plants, organs, tissues and cells.

In the description of plant-shapes the notion of L-systems may be applied. The notion of L-systems was conceived in 1968 by A. Lindenmayer as a formal model of plant development (Prusienkiewicz and Lindenmayer, 1990), and became also a part of formal language theory. All plant-shapes generated by one deterministic L-system are identical, so stochastic L-systems were introduced in order to produce more variation.

The drawback here is that, when a desired plant shape is given, one cannot find, in an algorithmic or otherwise mathematically precise way, an L-system that will generate it. The model we aim to introduce is that of an "abstract plant" i.e. not a virtual plant, where certain model-parts are controlled by suitable parameters. Plants are continuously communicating with an ever-changing environment where macro and micro effects interact. The parameters in the abstract plant enable us to measure the effect of these interactions; hence introducing parameters and choosing values for them with the aim of obtaining a virtual plant resembling a given specimen contradicts our aim of using the "abstract" plant in the study of the morphogenesis as a time sequence of adaptions to interval and external stresses acting on the plants.

Nevertheless virtual plants may be used in constructing an abstract plant as we will see. In this paper we provide the blue-print for the construction of (your own) abstract plants and provide concrete examples.

The structure of the paper is as follows:

1. Identification of the Problems
2. Outline of a Possible Solution (Multi-parameter Modelling)
    - 2.1. Abstract Plants : Geometrical Approach
    - 2.2. Abstract Plants : Materialization via Finite Element Methods
    - 2.3. Some Tools. NMR-imaging, Virtual Plants
3. Applications and Examples
    - 3.1. An Example of Geometrical Approach (The FRACPO Model)
    - 3.2. Supershape Optimisation in Plants
    - 3.3. Supershape and Finite Element Analysis
4. Conclusions

1. Identification of the problems

At first sight a plant appears as a combination of more or less regular geometric shapes continuously changing in time. This evolution involves deformations of already existing parts and the generation of new ones and it is characterised by rather obvious features of selfsimilarity, repetitivity and symmetries. Additionally the overall structure of the plant is self-avoiding i.e. no different parts can inhabit the same spatial-temporal habitat. Growth is the observed effect of interrelated internal and external forces acting in and on the plant. So in trying to understand the botanical laws governing plant growth we can study the effects of manipulation of external forces, (e.g. light, water, temperature, stress of any kind ...) on the growth of a type plant. All measurements lead to a lot of numerical facts or data that can only be integrated in a so-called "mathematical model".

Now exactly the fundamental features of self-similarity, repetivity and symmetry prompt the construction of a discrete model providing a sort of skeleton for the real plant. All of this, besides some fundamental botanical guiding principles, is purely mathematical or algorithmic in nature. The next step, i.e. realising the skeleton as a "real" plant by dressing it with tissue etc..., requires actual knowledge of its functional and structural botanical structure.

Computer modelling may be used in the construction of the plant skeleton, in such a way that enough degrees of freedom (parameters) are available enabling one to draw conclusions from the comparision of the abstract plant to the real plant in terms of predicted values for the structural parameters versus measured values. It is here that the different role of parameters in "virtual" and "abstract" plants is most clear.

We may list the problems we face, as follows:

1. To study the causes of the observed growth effects and to understand the interrelation of external actions and internal reactions.
2. To describe the internal logic of the deformations appearing in time, explained in terms of the biological material and the forces present on it or in it.
3. Globalizing to the whole plant the results of local analysis at different scales e.g. the micro-scale (cell-growth), the scale of plant-organs or parts, the time scale (short term or long range phenomena).

4. To predict the plant's reactions, at specific developmental stages, to a given situation. Obtain a visualisation of the important phenomena involved. For aesthetic reasons and perhaps for aesthetic reasons only (!) one may want this visualisation in 3-dimensions to be as close to reality as possible.
5. The model constructed should be "generic" i.e. free of in-built constraints depending on results or theories contained in those to be tested in the actual research.

In other words : "desired" solutions should not unconsciously be programmed into the model. If possible the model should be applicable to different plant-types up to changing only the function of the parameters used in the definition of the abstract plant, i.o.w. it can be used for trees but for cacti too! In the examples given below it will be clear how different features may play a more dominant role e.g. geometrical shape (Lamé ovals in bamboo), self-similarity and finite elements methods (root-growth, apical meristem).

2. Outline of a possible solution

The reduction of a rather amorphic surface or body to a more geometric object may be inferred from basic methods of Algebraic Topology, i.e. triangulation of a surface. This is nothing else but choosing points on the surface and connecting these by line segments so that a rough approximation of the surface is obtained. Using barycentric subdivision one may refine the triangulation further until a suitable one, close enough to the real surface in some mathematically precisable way, is obtained. Surfaces appearing in nature are usually represented by differential equations, although this is often already an approximation or a local description. Combining the idea of triangulation and analytical manifolds yields a concept that allows treatment by finite element methods later so it is an obvious candidate for the mathematical model. The following outlines a possible solution for the problems listed in Section 1.

2.1. Abstract Plants : Geometrical Approach

Find a discretization of the important differential geometry features, such as curvature, global curvature etc..., and develop a triangulation of the geometric objects suitable (!) for approximation of the desired geometric quantities (not every triangulation that approximates the surface within desired limits is good enough to approximate curvature or global curvature for example), as well as the dynamics of the whole system taking into account certain restraints stemming from boundary conditions (in our case growing conditions, growth limits, ...).

A lot of software has already been developed in Discrete and Differential Geometry, we may refer to an interdisciplinary project "Geometry of Subvarieties and Applications in Geosciences and Medical Image Production", by J.M. Morvan (U.C.B. Lyon I). Let us call the discrete approximation of the surface a **maze**. There are several software packages available for constructing mazes ("mailleurs"), some of these use finite element methods allowing to minimalize the errors in the approximation of a problem involving partial differential equations by numerical solutions obtained by the discretization of the domain.

Methods of **maze-optimization** allow to enhance local as well as global results. It is possible to develop mazes adapted to the local geometrical structure one wants to study e.g. curvature. There exists software for the construction and manipulation of triangulations called MESH and developed by J Hoffman and others. The latter has been extended by E Boix, so that the discretization procedure garantuees that the produced triangulations will represent in an optimal way the local geometry of the surface. This discretization therefore allows calculation of the associated geometrical quantities in an acceptable approximation.

The structure of the procedure allows to distinguish between the topological and the geometrical information from the triangulation. Automatic refinement and local modification of the maze is possible. The existing software has been tested at the U.M.R.-site CREATIS and applied to a simulation of the deformations of the heart-muscles. The possibilities of local modification allow a "dynamic" version of the maze and that would provide us with a satisfactory solution. The dynamic version characterizes evolutive phenomena starting from image sequences, and it involves segmentation, creating correspondences, fusion events and the modelization of the phenomenon in its space-time evolution.

For specific problems a more simple model may already yield satisfactory results (see FRACPO)! So the result of our approach to problem 1 is the creation of an **abstract plant** given by the maze-approximation of its geometric structure equipped with a number of relevant growth parameters characteristic for the type of plants studied In other words, we start from the idea that the plant strives for an "ideal form" the picture of which is printed in its genetic memory, the differences between this ideal shape and the real shape are to be explained by the adaptations to certain "external-factors" highlighting the failures making the plant's environment to be non-ideal. Experiments, botanical knowledge and intuition are the basis of the selection of the parameters to be used in the definition of the abstract plant (e g branching height, dimensions of plant parts and their ratios,...).

Selecting these essential parameters is therefore very delicate and the set of selected parameters should allow to obtain a blue-print of the plant. Here we stress again the difference with the construction of "virtual" plants We do not wish to adapt parameters so as to obtain the nicest pictures of photorealistic quality, we need parameters that allow us to describe the plants, study their behaviour under oriented experiments, and then explain the divergences of their values in different situations completely in terms of the functional and structural features of the plant! How external or internal forces act on the parameters can be calculated in the maze-model, the plant's reaction to it is measured in the experiments and the causality detected in this process is part of the solution of problems 1,2 and 3 listed in foregoing section We stress that this is obtained by "gauging" the abstract plant through well-oriented experiments that provide us with "expected values" for the parameters in the model.

For example, if the height of the first branching point is a parameter then we can have an idea about the "expected value" for this parameter by making a large enough number of representative measurements, when studying then a given set of plants a remarkable deviation of this parameter value marks the effect of an unusual influence (interrelations between parameters etc will lead to an understanding of this influence on the whole plant and show the direction for further investigations).

The gauging of parameters essential in describing the structure of the plant is what makes comparison to real plants possible and it is therefore very different from the choice of parameter values in order to obtain a reality-resembling shape

Indeed, in "virtual plants" the introduction of probabilities may produce less artificial looking images but it contradicts the fact that the growth process is determined by real forces the presence of which is everything but accidental Why a certain branch appears at a certain spot on a stem is exactly the object of study, so the reduction of this phenomenon to a probability is in fact completely ignoring the morphogenesis

It is also a correct gauging of the abstract plant that avoids the undesired inclusion of a-priori hypotheses in the model (e g if in the model it would have been accidentally built-in that the branching height is always more than the thickness of the stem, this would be detected in a carefully carried out gauging for the plant type under consideration) The foregoing shows that we can take care of problem 5 in Section 1 The abstract plant (i e all parameters in it) are in fact gauged at all essential stages of its development Therefore the "abstract plant" really becomes a spatio-temporal sequence of data, that can be visualized, calculated and manipulated, this makes it possible to use it as a solution for problem 4 in Section 1.

2.2. Abstract Plants : Materialization via Finite Elements Methods

The abstract plant of Section 2 1 is not much more than a sequence of geometrical and numerical data In order to put flesh on this skeleton the nature of the material building the plant has to be investigated However there is a weaker problem that has to be investigated first how do forces distribute themselves over the (part of the) plant considered? This problem does not depend too much on the nature of the material and it is more geometric-mechanic in nature.

Here again our discussion follows the ordering of the problems set forth in Section 1 For problem 1 we add to our existing model (i e a maze approximation of the shape together with a set of parameters and a list of their expected values obtained by "gauging" experiments) a Finite Element Analysis Due to the rapid development of digital computers, finite elements have become one of the major tools of designers and engineers The basic idea is to replace continuous functions by piecewise polynomial approximations, this idea goes back to estimations of π obtained by using regular polygons as approximations of the circle By letting the number of sides of the polygon grow one intuitively believes that the length of the curve converges to the length of the circle (a mathematician would like to see a proof first), so π can be approximated

The same method is used in engineering albeit with the help of powerful computers A number of excellent software programs are available to model FEM In principle the finite elements are highly comparable to the maze construction of the mathematical model But in Finite Elements forces can be applied to the structures which cause a deformation, depending on the material characteristics The stresses in any point of the mesh can be calculated and visualised on the total structure This allows to literally see the force flow through the object In plants FEM methods have been applied only sporadically notwithstanding the power of the method.

One of the most detailed analyses of pattern formation in shoots has been performed by Green and co-workers. Undulating surfaces in apices leading to the formation of leaves or other organs were suggested to be the result of minimal energy buckling (Green, 1992). Minimal strain energy configurations tend to avoid sharp curvatures and small random fluctuations in shape (tunica e.g.) are preferentially amplified to give a multipeak final arragement. The mechanism proposed to be involved in such rearrangements are the cellulose microfibrils of the cell wall.

To refine the model FEM shell elements (FEM can be modelled on virtually any shape or structure, rendering the link with a topographical approach a rather natural one) were used with apex shapes of *Vinca minor* and data for wall thickness and mechanical properties of the cell wall material for this plant species. Their findings suggest that in the regions where new leaves will appear at first as visible humps, shear forces are high, while in the plane of the surface the tension is low (Selker *et al.*, 1992). They clearly showed that biophysical processes are involved in the construction and patterning of leaf primordia, and identified a continuous cycle for apex morphogenesis as a temporal sequence.

In trees FEM was used to study growth and growth responses to external factors (Matteck and Kubler, 1995) Trees react to environmental stresses by a number of mechanisms, mainly based on the presence of cambial layers. These meristematic layers allow the plants to reinforce the material when necessary. For example when a plant is injured or leaning on a rock, typical reaction wood will be developed by the tree at very specific sites. After careful observations of a large number of trees, mainly aberrant growers, Matteck and co-workers developed FEM for wood and trees. This led to the precise identification of the types of reactions, all pointing to a minimization of stress.

Since we have a triangulation of the geometric shapes we are studying at hand via the maze model, it is now easy to develop finite element analysis on it. Now that deformations can be completely visualized, in fact the dynamic process can be completely visualized in a very realistic animation, it remains to explain the phenomena in terms of the internal logic of the plant structure. Here one has to start from basic axioms, physical principles in mechanics, principles of chemical reactions,... Most of this is outside the scope of this note. Let us point out that we may compare our problems to existing software and applications. The dynamic maze model used in modelling stresses in the heart-muscles can be adapted to the plant-situation e g to the study of fluid-flows.

The model applied to the study of deformations of a surface under tension and geological forces is **abstractly** similar to a model for the deformation of the apical meristem in the vegetative or flowering case, or also for the deformation of a disc-like section of a bamboo node Starting from a minimal set of axioms and principles, taking into account the numerical specifications reflecting the structure of the plant material, the dynamical maze-version of the abstract plant enriched with the dynamical version of the finite element analysis, provides us with a tool allowing to solve problems 1,2,3 in Section 1. Note that a finite element analysis at any given moment in a growth process is depending on the geometrical shape at that moment; therefore the possibility of using both the finite element analysis and the geometric approximation in a single dynamic model depends completely on the fact that the maze model could be constructed (and this is done automatically by the software) so that the essential geometrical characteristics are controlled by the discrete model, in the time process!

Again, the use of the model in the solution of problem 4 of Section 1 is made possible by experimental "gauging" and obtaining "expected values" of the parameters of the system (now including the finite element analysis) as explained before. In this way it is probably possible to study the forces created in the plant by pruning and to predict the plant's reaction to it. The addition of the finite elements analysis to our "abstract plant" does not change its generic qualities (problem 5 in Section 1). An abstract tree will be different from an abstract cactus but the mathematical model underlying both is the same (now including the finite element analysis) and the difference is created by the role and the gauging of the parameters!

2.3. Some Tools. NMR-imaging, Virtual Plants

We have at times pointed out how an idea of an "abstract plant" differs from the concept of a "virtual plant". However the latter may certainly be used as a basis for the construction of the former. In the FRACPO model (see 3.1.) the geometric information about the plant-shape may be much more crude than what is proposed in the geometrically very detailed maze model we treated in this and foregoing section. So even a crude virtual plant model may be equipped with suitable parameters and then turned into an abstract plant in a sense.

Even more directly one may input living plant cell or tissue specimens in 3-dimensional digitized format. There now exists a number of non-destructive approaches. Such non-invasive techniques have the major advantage that they may be used to "measure" the image over several periods of time. For plants and even large trees, students of virtual plants have used digitizers based on sound, light or magnetism (Room *et al.*, 1996) and even lasers. Software developed for such digitizers is available via internet

For smaller specimen non-invasive studies have become particularly attractive with the introduction of confocal laser scanning microscopy. The laser scans very precisely in planes inside cells or tissues, imaging software allows to produce 3-dimensional images. In this way even 3-dimensional images of individual chromosomes have been produced.

For larger specimen proton nuclear magnetic resonance imaging proved to be very promising. This technique had been used in medical diagnostics and has then been applied to plants to study the structure of red raspberry, gooseberry, strawberry fruits and grapes and developmental changes in ripening fruits of red raspberry and grapes (Goodman *et al.*, 1993, 1996; Williamson *et al.*, 1992, 1994).

3.1. An Example of Geometrical and Fractal Approach (the FRACPO Model)

Symmetry and self-similarity observed in plants were the characteristics at the origin of the idea to develop discrete methods; the repetition of certain shapes in the overall plant structure suggests an iterative principle. It is therefore natural that fractals should appear as possibly useful geometric structures. However the characteristic phenomena in fractal geometry depend on an awkward interplay between infinite quantities or objects and finiteness conditions e.g. of the area where processes are taking place (Peitgen and Richter, 1986).

Even though fractals may be used to approximate geometric shapes of almost arbitrary complexity, there are other methods of discretization (as pointed out in 2) probably more suited to dealing with concrete problems of plant morphogenesis. This leads to the idea of defining a kind of finite model of a fractal, based on iterated self similarity, but then we rediscover some special L-systems.

The basic concept in L-system theory is that of "rewriting". This is a technique for defining complex objects by successively replacing parts of an initial object by a copy of a "generator" object. The rules governing these replacements are called **rewriting rules**. The snowflake curve is a simple example (Prusinkiewicz and Sandness, 1988). The best understood rewriting systems operate on character strings. This enjoyed considerable interest after Chomsky's work on formal grammars in the late fifties. In 1968 A. Lindenmayer introduced another type of string-rewriting mechanism, later termed L-systems. The novelty here was that the rewritings (sometimes called productions) are not necessarily applied sequentially but in parallel i.e. one may simultaneously replace all original "letters" in a given "word". Productions were now intended to capture cell divisions in multicellular organisms so many divisions may occur at the same time. Deterministic and context free L-systems, so-called DOL-systems, were intensively studied and it was shown that DOL-systems could generate fractals (Prusinkiewicz and Hanan, 1989).

There are also parametric OL-systems but we refer to the extensive literature for detail on these matters (P. Prusinkiewicz and A. Lindenmayer (1990) *The Algorithmic Beauty of Plants*, Prusinkiewicz 1994, Prusinkiewicz *et al.*, 1993, 1994). As explained in parts 1 and 2 of Section 2, our approach replaces the L-system by a maze-model of the geometrical object but L-system aspects may be present in the description of the maze-model of plants (whereas they are not present in the general theory of discretization of differential geometry). As we pointed out before, several simplifications may be possible, we may reduce the maze-model to a linear construction, with line segments representing branches of a tree for example, and to this level of approximation the maze-model and the L-system approach coincide as far as the construction of the skeleton of the plant is concerned.

Let us give a simple example just to explain the main ideas. In figure 1, t_1 is the time until when the tree has the shape B_1, t_2 is the moment up to when the tree has the shape B_2 etc... The form of the tree is the "generator" that will be iterated. Based on phyllotaxy it is possible to make a finite list of possible 3-dimensional "generator" parts sufficient to generate any existing real tree.

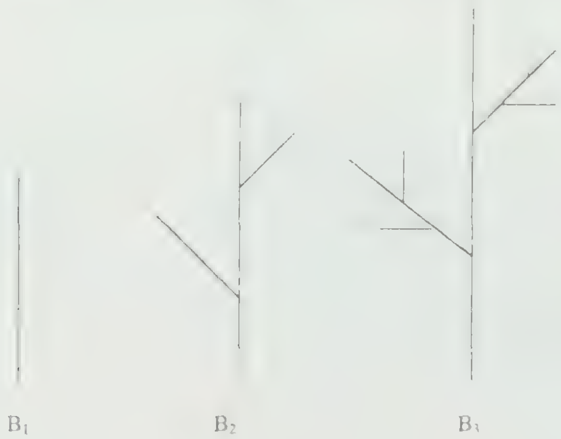

Figure 1

Given the iteration rules and the selected generator, the computer can draw pictures of the branching model of the tree at any time $t = t_n$. We talk about a **tree of type** b where b is a generator selected from the list of branching types. It is possible to construct trees of a combined type by allowing different types appearing in a time sequence, but let us focus on one constant type here. Now t_3 is the ultimate moment when the tree of type B_2 looks like Figure 2. At $t = t_3$ there are three parts, resembling the tree at time t_2, this of course corresponds to the fact that there are 3 branches in the generator B_2.

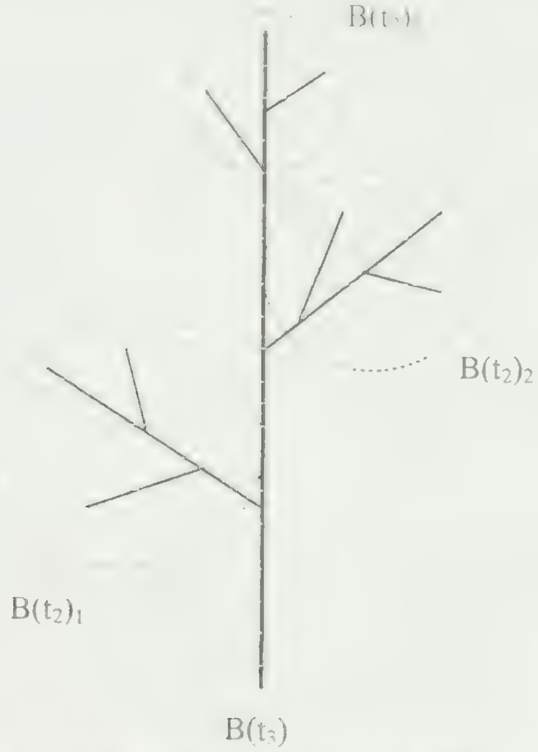

Figure 2

At $t = t_4$ there will be 3 parts looking like the tree at time t_3, 9 parts that look like the building block B_2 (up to size, but this is one of the parameters to be introduced later), all together it has then 27 branches (including the main stem). If m is the number of branches (including the stem) of the generator then at time $t = t_n$ there will be $m^{(n-1)}$ branches in the tree and they are ordered in $m^{(n-2)}$ subtrees of type b, m subtrees of type given by the tree at time $t = t_{n-1}$.

The time t_2-t_1 is necessary to go from form I to the form of the generator and this time does not need to be equal to the time t_3-t_2 necessary to go from type b to the next iterated form. The fractation time sequence is: (t_2-t_1/t_1; t_3-t_2/t_2-t_1; t_4-t_3/ t_3-t_2;......). After a certain number of steps the tree steps growing according to our principles (a good estimate for this is given by $\log_m$: number of branches in a fully grown tree). If we assume that the time for refractation grows linearly at each reproduction step, this comes down to the fractation time sequence being constant, say $F_t = t_2$-t_1/t_1 (more generally one may take an average for F_t.) The axiom $F_t > 1$ is a natural one. There is a recurrence formula $t_n = t_{n-1} + F_t^{n-1} t_1$. When $F_t^{n-1}.t_1$ is too long to wait for (say bigger than 20 years) then we consider the growth of the tree finalized at the moment $t = t_{n-1}$, so its final form has $m^{(n-1)}$ branches. In order to reintroduce real dimensions in the picture obtained so far we have to introduce (size-) parameters that will define our abstract tree as in Section 1.

Note: this is a simplification, further parameters will be necessary in a complete study

Size Coefficients

Write B_n for the tree-form at time t_n, put $B_n = \alpha_1^{(n)} B_{n-1} \oplus \ldots\ldots \oplus \alpha_{mn}^{(n)} B_{n-1}$ where the $\alpha_1^{(n)}, \ldots\ldots, \alpha_m^{(n)}$ are coefficients relating the size of the parts looking like B_{n-1} to the size of the original B_{n-1}. At $t = t_1$ we introduce the parameters h_1 = height of branch and Δ_1 = diameter at base. At $t = t_2$ we introduce the m size coefficients $\alpha_1^{(2)}, \ldots, \alpha_m^{(2)}$ and look at $B_2 = \alpha_1^{(2)} B_1 \oplus \ldots\ldots \oplus \alpha_{m2}^{(2)} B_1$ where m_2 m given by the branching type of the construction and $\oplus$ means the composition of the tree B_n by its parts looking like B_{n-1}. This looks like

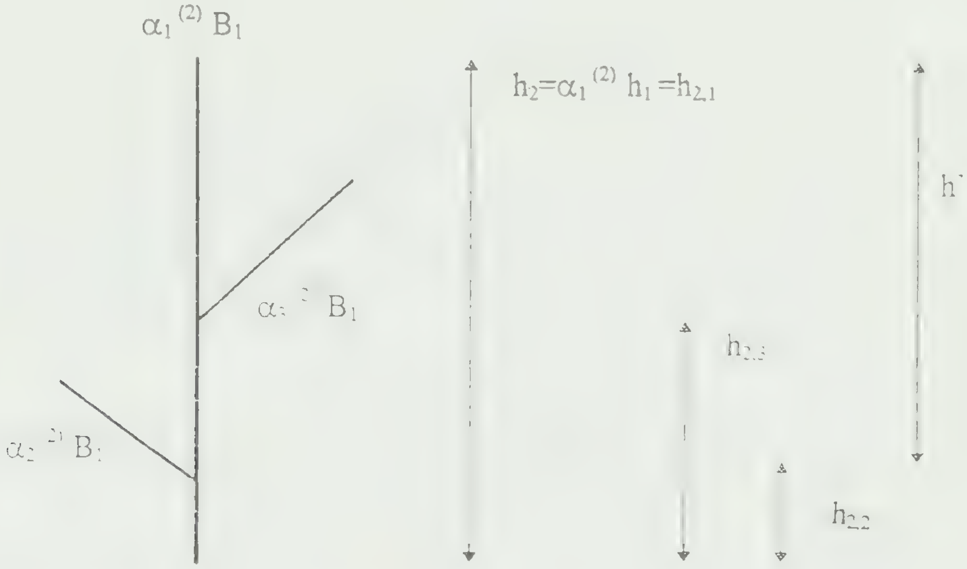

Figure 3

These coefficients determine length and diameter of stem and branches; it is natural to demand $\alpha_1^{(2)} \geq \alpha_2^{(2)} \geq \alpha_3^{(2)}$. A parameter often introduced (e g. in the classification of savannah plants) is the branching height or the height at which the new shoots appear on the plant i e. $h_{2,3}$ in the picture. At the branching point the diameter of the branch should be less than (or equal to) the diameter of the stem. Under abstract undisturbed growth conditions one could expect that $h_1/\Delta_1 = h_2/\Delta_2$, where Δ_2 is the diameter of the first branch in B_2. The foregoing condition then may be expressed as:

$$\alpha_2^{(2)} \Delta_1 \leq \left((\alpha_1^{(2)} h_1 - h_{2,2}) / \alpha_1^{(2)} h_1 \right) . \alpha_1^{(2)} \Delta_1$$

or $\alpha_2^{(2)} \leq \alpha_1^{(2)} - h_{2,2}/h_1$. In a similar way one obtains : $\alpha_3^{(2)} \leq \alpha_1^{(2)} - h_{2,3}/h_1$. Let us assume that for branches of the same type (e g those looking like B_1 in the picture of B_2) the ratio of diameters (branch-stem) is a constant. This condition may then be expressed as $\alpha_2^{(2)} / (\alpha_1^{(2)} h_1 - h_{2,2}) = \alpha_3^{(2)} / (\alpha_1^{(2)} h_1 - h_{2,3})$.

Hence $h_{2,3}$ may be calculated from the other data. Similar arguments may be used at any t t_n, so the assumptions we have made entail that only the first branching height has to be considered as a new parameter (the other heights may then be calculated from it and the size parameters).

Now B_n is composed of parts looking like B_{n-1} with suitable size coefficients that may in principle be all different However, if we carry the fractal-based philosophy further, i.e. use the notion of iterated self similarity in full extent, then we should view $B_n = \alpha_2^{(n)} B_{n-1} \oplus \ldots\ldots \oplus \alpha_{mn}^{(n)} B_{n-1}$ where we then may assume $\alpha_2 = \ldots\ldots\ldots = \alpha_2^{(n)}$, $m_n = m$, $\alpha_m^{(n)} = \alpha_m$ for every n This requires a small modification in the description; this can be seen clearly by looking at the following picture for B_3

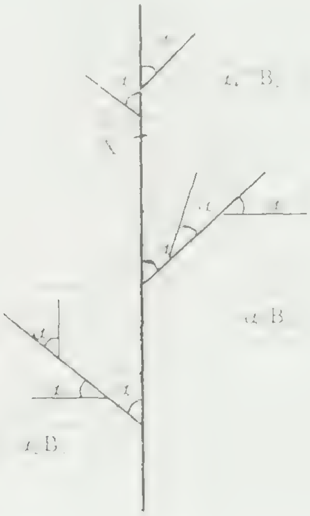

Figure 4

The parts of B_3 looking like B_1 that are not part of a B_2-type part have been taken together in a new part that looks like a B_2 by introducing X on the stem and viewing the top part of B_3 (above X) as a new copy B_2 with size factor $\alpha_4^{(3)}$ If one again assumes that the diameters at the branching points have a ratio exactly inverse to the ratio of the heights of the branching points, then the position of X can be calculated from the total length of the main stem. After years of growth one has to change this rule as the diameter of stem will not expand so linearly anymore, also the branching angles may be varied etc

We end up with a model determined by size coefficients $(\alpha_2, \alpha_3, \alpha_4, \ldots)$, branching heights h_{2n}, stem-coefficients β_n = (height at t_n) / h_1 for every n, completed by the branching type (B_2) Note that the geometrical shapes are by assumption all cylindrical (given by diameter at base and height) so we did not have to start from a more complicated maze model as described in Section 1 Of course this example is a simplification, it would not allow a real local analysis of geometrical nature, nevertheless it is quite useful in describing patterns of tree growth and capable of producing realistic looking "forest growth" pictures and animation.

We include the following short description of the FRACPO-model (Chen *et al.*, 1993, 1994a, 1994b, 1994c, 1996).

DESCRIPTION The FRACPO model is a three-dimensional crown model that has specifically been developed to generate individual poplar tree frames within a stand (Chen *et al.*, 1994a) The model has been based on experimentally observed and simplified growth rules of young poplar trees grown under Short Rotation Forestry (SRF) practices, and uses a fractal approach Stems and branches are considered as the structural carriers of leaves, and the spatial distribution of leaves is mainly determined by the structure and architecture of stems and branches.

Several branch characteristics have been measured in detail on young poplar trees grown under SRF, and a computer graphical model was developed to simulate the dynamics of crown architecture.

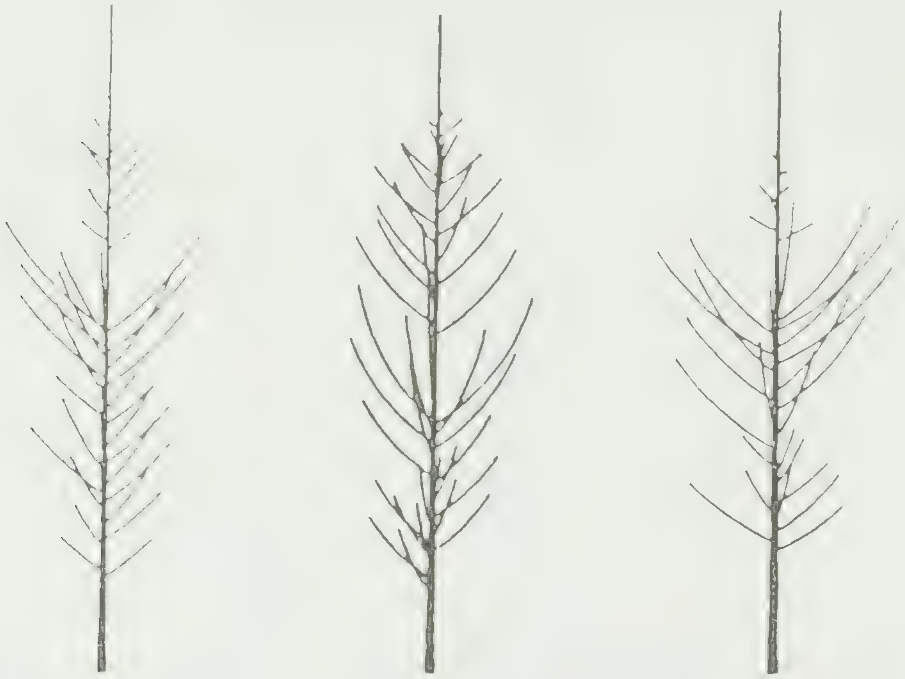

Figure 5

The structural crown development is then coupled with a leaf submodel to generate and simulate the three-dimensional leaf distribution in the canopy. The leaf submodel primarily integrates information on leaf initiation, leaf longevity, expansion and distribution of leaf sizes within the canopy (Chen *et al.*, 1994b and 1994c).

These parameters determine the instantaneous leaf area index (LAI) and the temporal variation of LAI. The spatial distribution of leaf area depends on the positioning of leaves within the canopy and on the variations in leaf size with their positions on stem and branches. The resulting three-dimensional leaf distribution within the canopy can then be used for calculations of canopy radiative transfer, light interception and photosynthesis.

INPUT: As described above and because of its fractal nature, the FRACPO model requires quite a number of detailed input parameters such as *(i)* length and number of internodes for each height growth increment (HGI); *(ii)* length, number and dimension of sylleptic and proleptic branches, *(iii)* successive branch growth increment; *(iv)* angles or origin and termination of branches, *(v)* insertion height of branches and relationship between height of insertion and branch length, *(vi)* leaf longevity, leaf size, leaf area and distribution of leaf area over branches, *(vii)* leaf area index as a function of the time of leaf initiation.

TIME STEP: The minimum time step in the time period required for the formation of one internode (i.e. 3 to 4 days).

OUTPUT

1. Dynamics of three dimensional distribution of leaf area density
2. Profile and top-down projections of the canopy
3. Distribution of light irradiance of leaf surfaces (combined with a three dimensional radiative model, CANLIP).

ASSUMPTIONS: To simplify the descriptive rules and processes of branching, internode formation etc., a considerable number of *assumptions* had to be made in the model. With regard to sylleptic branches, for example, it is assumed in the model that all sylleptic branches start to develop only in the middle of the growing season. Furthermore, they are produced acropetally, but not continuously. And finally, several sylleptic branches arise from the preformed buds in one surge of growth. Other main assumptions in the model include: *(i)* the time period required for the formation of one internode is identical for all internodes, *(ii)* the average length of a branch growth increment is a fraction of that of the previous one, *(iii)* the average internode length within a branch growth increment of order *k* is a fraction of that of the previous order *k-1*. This means that the sequential pattern of internode length in a branch growth increment is a symmetrical trapezium, *(iv)* branch curvature is constant, or in other words the branch angles do not vary with height or height growth increment, *(v)* the timing of leaf initiation is the same as its associated internode formation.

SPATIAL and TIME SCALES: Spatial scale: from crown to tree and to stand level. Time scale of output : from 3 or 4 days to output over the entire growing season.

APPLICATION: The model can be used to study the distribution of canopy gap fraction by means of a digitized image analysis system. The relationship between branch angle and leaf angle can also be examined with the FRACPO model. Top-down projections of different layers of a simulated canopy can then be used to estimate the downward cumulated LAI (Figure 6). Furthermore, relationships between branch angle and leaf orientation or between branch orientation and spacing density can also be examined with the model (Chen *et al.*, 1996).

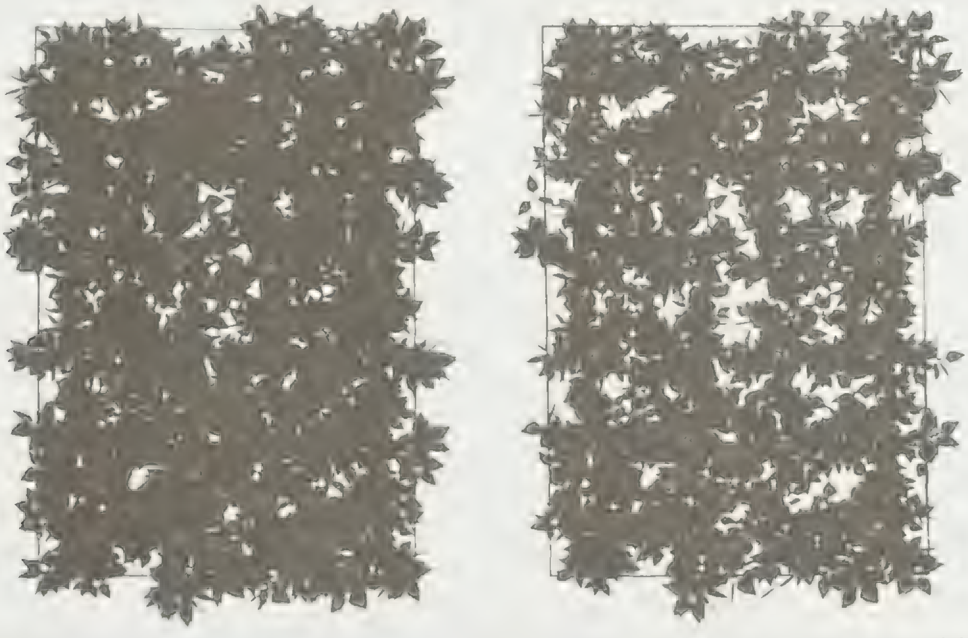

Figure 6: Projection of the whole canopy (6a) and of upper and middle parts of the canopy (6b) of the simulated stand on Day 230 (Chen et al., 1993)

Application of fractal theory to tree crown architecture:
Tree crown architecture and function are of crucial importance to overall tree growth and development. Studies describing tree crown architecture not only pay attention to the modular structure and the static architecture modelling approach, but also focus on the dynamics of the tree architecture and of the module. The latter approach then results in the tree architecture generation models. It has already been shown (proven) that the theory of the fractal geometry (advanced in the 1970's) has a significant potential for the study and the description of tree crown architecture and tree forms. Several studies (Strad, 1990; Zeide, 1991; Zeide and Pfeifer, 1991) have already used the fractal geometry approach to (synthetically) analyse the relationship between the tree growth allometry and crown architecture. Some authors (Ye *et al.*, 1993) even claim that the fractal dimension of a tree crown is the best criterion for the definition of the optimum tree crown architecture. Based on this optimum crown architecture, the hypothesis could be forwarded that trees should develop (produce) the largest crown area or crown surface by spending a minimal architecture cost in a limited growth space. This would mean that the only way for a tree to increase its crown surface area is to increase its fractal dimension, provided that overall crown volume of the tree remains the same. So, the 'economical' (cost versus benefit) and the effective characteristics of crown architecture could be evaluated using the fractal approach.

The fractal geometry approach is one of the better ways to get a better insight in the growth patterns of trees. The fractal geometry approach can provide a useful tool for tree breeding and forest silviculture approaches (Figure 7).

3.2. Supershape optimization in plants

In nature a lot of space is available but nevertheless it costs energy to occupy space, in particular to maintain a certain state of "being" in this space. The strategy of growth is therefore necessarily one of **invading a space** and trying to stay there. Therefore the plant that wants to grow needs to develop parts (e.g. roots, branches, leaves) that penetrate the neighbouring space, the shape of these parts will therefore correspond to the different mini-max type of problems imposed on them by internal and external forces. It is clear that pointed shapes facilitate penetration in newly conquered space so it is no wonder that the plant's pioneering parts like rhizomes, roots, buds, etc... often feature rather pointed shapes.

Once a certain space can more or less safely be occupied, pointed shapes are too fragile, so with the aim of minimizing cost in energy certain plant parts will maximize the amount of space occupied within certain limits. Another advantage of maximizing space utilization is that the resistance of an object towards mechanical forces may grow with the area of the object if a suitable form is obtained. A kind of shape that arises naturally is the so-called **supershape** based on Lamé ovals.

A first example of a supershape may be obtained by inflating a balloon within a box having a cube-shape. The balloon will be roughly spherical until it touches the walls, when inflated further it will assume a shape with flattened parts and it will become increasingly harder to inflate it further (but this is solely due to the material-properties of the balloon).

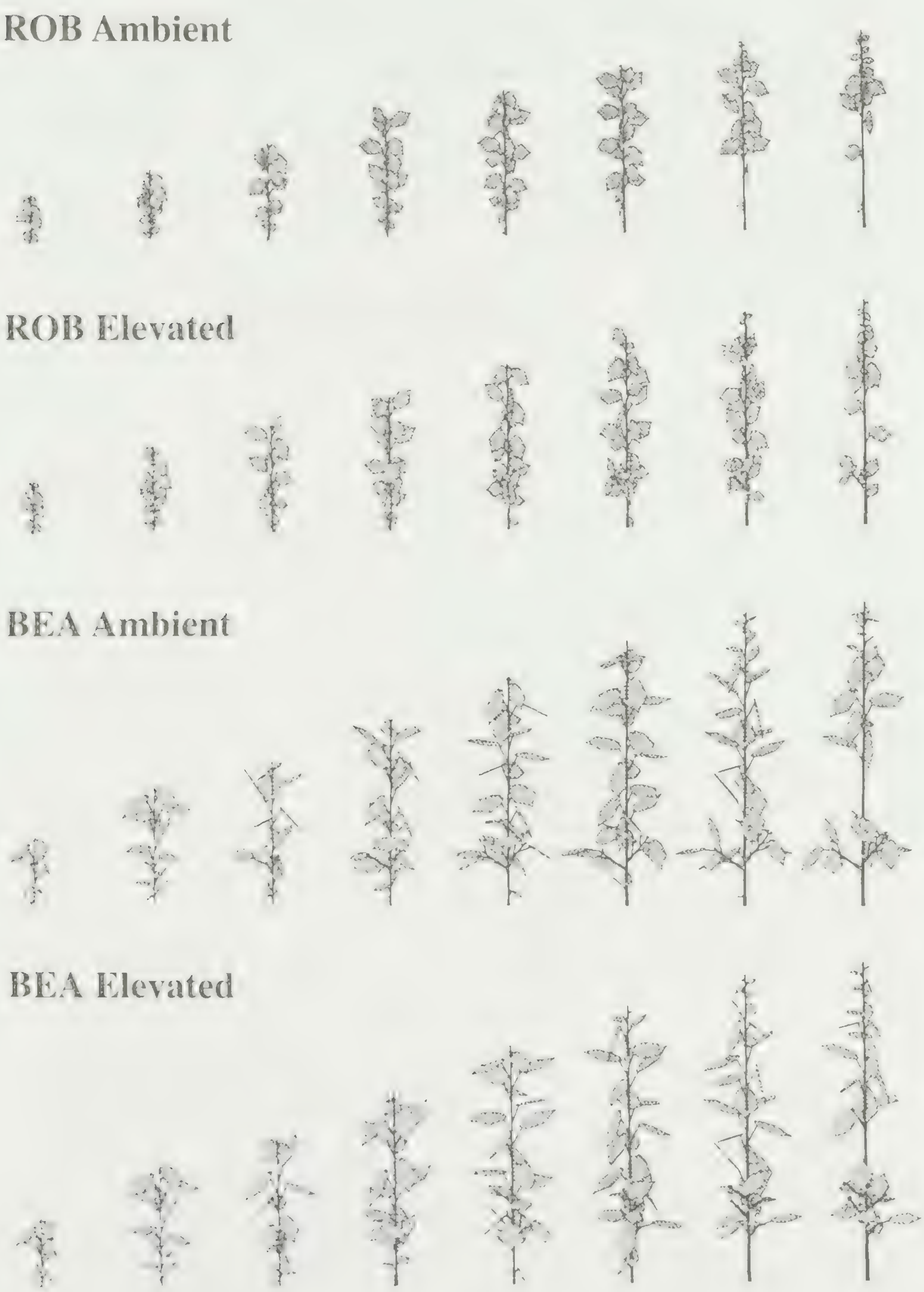

Figure 7

A graphical comparison of poplar clones "Beaupre" (BEA) *and* "Robusta" (ROB) *grown in ambient (+/- 350 μmol mol^{-1}) and elevated (ambient + 350 μmol mol^{-1}) CO$_2$ concentrations.*

In a way supershapes are opposite to triangulations of surfaces because the supershape approximates the surface of a usually convex polyhedron. Of course we are not forcing plants to grow in boxes, the fact that supershapes appear so frequently must be due to the fact that external forces acting on the growing plant parts do constitute a more or less regular force-field limiting the plant's free space. The stronger the forces the more rigid this "frontiere" for the plant's free movement will be and then supershapes will appear as if the plant is pressing against the now invisible box-walls. Most striking examples of supershape, presenting us with plants having an almost perfect Lamé ovoid shape, are found amongst stem succulents and in some pachycaul trees. Examples are *Dolichos*, *Jathropha berlandieri*, *Calibanus hookeri*, *Pachypodium brevicaule* and species of *Nolina* or *Beaucarnea*. The resistance encountered by these stems, that may obtain an almost globular form in some species (like *Jathropha berlandieri*) obviously causes the creation of the supershape completely in correspondence with our assumptions.

A large number of plants have stems with a square (or almost square) section (mainly associated with decussate phyllotaxy). Good examples are members of Lamiaceae and Melastoma-taceae, e.g. *Tibouchina*, and the square bamboo *Chimonobambusa quadrangularis*. Other so-called square bamboos are grown artificially by placing a mould over a circular young shoot which is then forced to grow in a supershape, such stems have a high ornamental value in China and Japan (Gielis, 1996).

Bamboo culms and rhizomes are hollow (except for *Chusquea* species) as are stems of several other grasses, sedges and *Equisetum*. Hollow internodes have the advantage of economizing on material while in fact optimizing mechanical resistance (Niklas, 1992). The specific forms of cavities or hollow parts can have additional interesting features, both mathematically and from an engineering point of view. For example in bamboo culms the hollow space of internodes is intermediate between an ellipsoid and a beam having a typical supershape. During initial elongation of the bamboo culm cavities are formed in cylindrical internodes, that this is really unavoidable may be understood if one realizes that bamboo culms have been recorded to grow 121 cm per day! Whereas regular Lamé ovoids thus arise in the form of the cavities in bamboo culms most often irregularities of different kinds occur e.g. caused by forces exerted on the local plant surface by formation of axillary buds or branches. As a typical case we mention the deformation of the nodal diaphragm which is comparable to the minimal energy buckling in tunica layers (Green, 1992).

In the beginning of this section we argued that supershape originates from the plant's reaction to external resistances when it is invading new territory, in the foregoing arguments we pointed out that supershapes also occur inside the plant so there must be different reasons for that. In fact we may learn a lot from a story about architecture. A rectangular place in Stockholm had to be rebuilt and the architects wanted to optimize traffic around the place from incoming roads. When the roads were planned along the houses in a rectangular form, the remaining space in the middle would be most suitable for the shopping centre that had to be constructed there, but the traffic would not move smoothly at all. The solution proposed by the engineer Piet Hein form Denmark was a Lamé oval and it turned out that traffic around the place gained about 30 % in efficiency.

We conjecture that specific Lamé ovals optimize the current or flow around the shape (and we were inspired by seeing Lamé shapes appear in Benard instabilities in hydrodynamics). Clearly, vascular bundles will have the smoothest trajectory following the contour of a suitable Lamé oval and fluid flows would be speeded up considerably (an important feature for a plant that grows as fast as some bamboo species). Before we summarize the characteristic effects of the plant's use of supershapes in its architecture let us provide just a little more insight in the mathematical properties of Lamé ovals.

For real numbers a,b the Lamé oval $O_n(a,b)$ is defined by: $(x/a)^n + (y/b)^n = 1$. Clearly for $n = 1$ this is just a line $bx + ay - ab = 0$, passing through the points with coordinates $(0,b)$ and $(a,0)$, i.e. the descending diagonal in the rectangle (Figure 8).

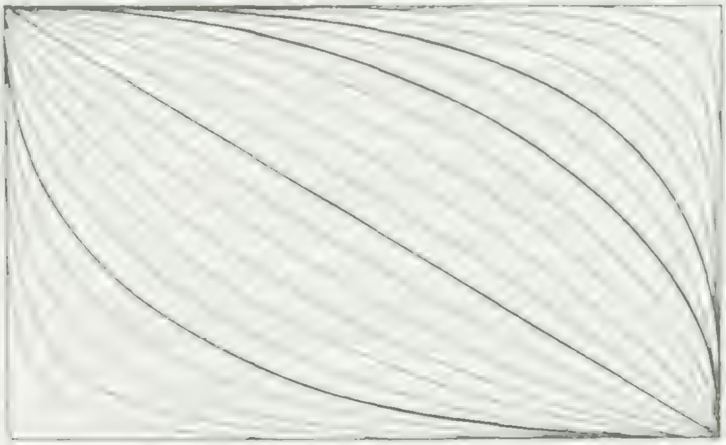

Figure 8

For $n < 1$ the curve is cusped, we will not consider these cases here. The case $n = 2$ is the ellipse (the circle in case $a = b$) and it is the only Lamé oval having foci, that is of course because it is a conic, or also the only Lamé oval having a finite radius of curvature at every point of the curve. Studying the cusps of the evolute curve one may observe that for $n < 2$ these cusps are embedded in the curve itself and thus there appear cusps in the axial points. For $n > 2$ the cusps are at infinity so the Lamé curve exhibits a Flachpunkt in these points. This means that the Lamé ovoid has a Flachpunkt too and can be theoretically balanced on an axis in a state of equilibrium.

Exact calculation of coordinates of points on a Lamé oval or ovoid may sometimes be impossible; this can be observed by taking $n = e$ (the natural logarithm basis) and then all points not on the x-axis or y-axis but on the Lamé oval have transcendental numbers for their coordinates. These mathematical oddities mean little or nothing for the applications we have in mind.

Let us summarize the aspects of "supershape" that seem to be meaningful in plant architecture:

- S1. The existence of a Flachpunkt provides extra stability.
- S2. The smooth trajectory around supershapes seems to optimize currents or flows around the supershaped object.
- S3. The supershape also seems to divert stresses over the object in a maximally efficient way.
- S4. Supershape cavities allow economizing on material while optimizing mechanical strength.

All of the aspects mentioned above, which we want to interprete as rules, have been observed in the study of bamboo, the supershape plant by excellence! But there is much more evidence for the foregoing "rules" and of course one expects finite element analysis to provide strong arguments to prove or to support the validity of the proposed "rules".

3.3. Supershape and Finite Element Analysis

The supershape aspects S2 and S3 in the foregoing section relate the appearance of the supershape to the stress-distribution over the surface of the plant-part. This was already very clear for some internal forces e.g. the cytoplasm of cells strives for as large as possible cell-content within the limitations of the cell wall because of the turgor pressure (like the balloon inflated in a box) This leads to a Lamé oval form and is responsable for the "rounded corners" of the cell.

With respect to external forces roots must provide pertinent examples of supershape because it is the root that has the unpleasant task to penetrate the soil. And indeed, roots of various plants do provide an example of a functional Flachpunkt. In figure 9 a schematic drawing of a corn root is presented, in fact this type of root is found in most grasses. The distal root tip can be approximated as a paraboloid of revolution. But proximal cell layers will increasingly exhibit a flattening of their "tips", leading ultimately to the Flachpunkt, just distal to the quiescent centre QC of the root.

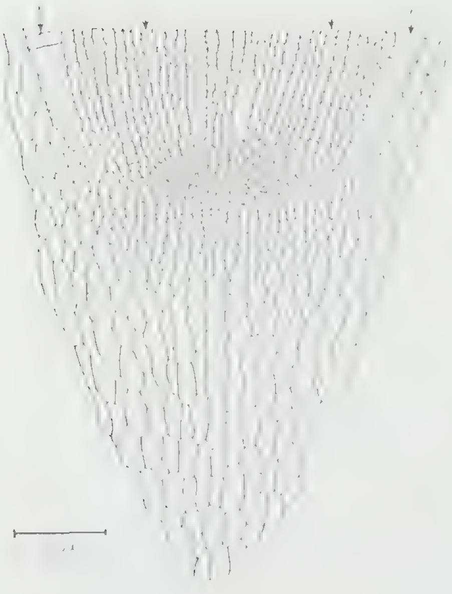

Figure 9
Median section of root of Zea Mays
(quiescent centre shaded; E, epidermis;
S outer layer of stele. From Clowes, 1961)

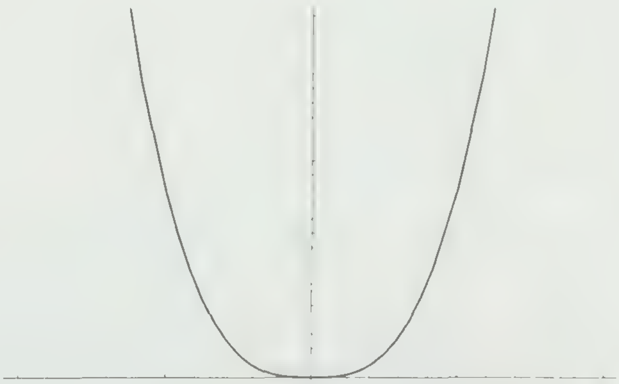

Figure 10
Flachpunkt of the equation
$(x/_2)^3 + (y/_{100})^3 = 1$

From an intuitive point of view the supershape of cell layers seems to have two main functions. One is to act as a drill during root growth, drilling the root cap deeper into the soil. The other function seems to divert stresses resulting from penetration into the soil, from the QC.

In a study using finite elements the mere shape of root tips was taken as starting point, in a first step neglecting differential mechanical features of the tissues involved. When a paraboloid is loaded with shear stress mimicking the forces experienced by the root cap when penetrating the soil two results stand out. One, the cap or Kappe which is easily distinguisable from the Körper in grass roots (Clowes, 1961) is deformed in such a way that the upper parts of the Kappe act as jaws, clamping to the Korper in its upper regions. Second, in the middle part of the paraboloid a low stress zone can be observed (Beirinckx and Gielis, unpublished results).

From our study it seems that the mere geometrical shape of cell layers in real roots also leads to the logical existence of a QC. The combination of the jaw-like deformation of the proximal parts of the root cap and the supershape of the Körper, results in an efficient drilling machine, while protecting its inner tissues. The Flachpunkt of the distal layers of the Körper seems to divert stresses away for QC when roots grow. This results in a low stress zone, indicating that biophysical and bioengineering aspects are involved in the establishment and the maintenance of the QC.

While roots are quite straightforward objects for study, and the genetics of root development are increasingly being unravelled (Sheres *et al.*, 1996), the precise role of the QC remains somewhat enigmatic today. A physiological study recently related the abscence of cell division in the QC to the presence of high levels of ascorbate peroxidase compared to surrounding tissue, leading to lower levels of ascorbic acid, a crucial biochemical component in the cell cycle (Kerk and Feldman, 1995). Quiescent centres are also the result of pure mathematical approaches of root growth. When growth of root apices was modelled using tensors, a specific type of growth tensor led also to mathematical existence of a QC (Hejnowicz and Karczewski, 1993).

The precise way that roots penetrate into soils has also been the subject of an interesting theoretical study using Finite Elements. Faure (1994) studied the stress field developed into different types of soils when roots grow into these soils. The FEM method led to a better understanding of the interaction between roots and soil. As the roots grow plastic zones appear around the tip, and the size of this zone is very specific for the type of soil. Alternatively the specific root form is also dependent on the type of soil. The developed method simulates actual root growth without using the analogy of the penetrometer action.

4. Conclusions

Recent researches on morphogenesis and development in plants, cells and tissues, mainly focus on morphology, physiology, genetics and molecular biology. In each of those fields impressive progress has been made and especially integrated approaches are very rewarding.

Biophysics (including bio-engineering) and bio-mathematics (both fields are traditionally a weak point of biologists) provide very powerful methods to understand more fully plant architecture and developmental processes. In this paper we have tried to summarize our vision on plant modelling. L-systems, the FRACPO model and fractal approaches, and supershapes are attractive but basically simple mathematical techniques.

In the rapidly developing field of computer graphics, real time modelling of development in plants, tissues or cells, is not a far away dream anymore. Increasingly these techniques will be integrated in any field of botany. But basic understanding of some mathematical techniques is useful.

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Genetic aspects of leaf development

Mieke Van Lijsebettens, Jonathan Clarke[1], Rudy Vanderhaeghen, Gerda Cnops, Martine De Bleeckere, Xuan Wang, Tom Beeckman[and], and Marc Van Montagu

Laboratorium voor Genetica, Department of Genetics, Flanders Interuniversity Institute for Biotechnology (VIB), Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium;
[and] Laboratorium voor Morfologie, Systematiek en Ecologie van de Planten, Universiteit Gent, B-9000 Gent, Belgium, [1] Present address: Department of Molecular Genetics, John Innes Centre, Norwich NR4 7UH, United Kingdom

Introduction

In view of their relative immobility, plants have evolved into organisms with distinct patterns of growth and morphogenesis. Intrinsic to these are autotrophic growth, converting light and atmospheric carbon dioxide into energy and nutrients, totipotency of all cell types, and organ development out of meristematic zones that are active during the entire life cycle. Processes of cell division, polar and non-polar cell expansion, and differentiation lead to patterning and the formation of organs with inner and outer architecture adapted to their specific functions. Insight into the molecular mechanisms that direct and control the different steps in the ontogeny of organs progressed a lot with the identification and study of crucial genes and their genetic manipulation in transgenic plants. Mutagenesis of the genome is a powerful tool to identify genes coding for regulatory, enzymatic or structural proteins with an important role in plant morphogenesis and provides the basis for gene cloning in this research.

We are studying the genetic control of leaf lamina formation using the weed *Arabidopsis thaliana* as an experimental system. This plant has been widely accepted as a model to study the molecular basis of plant growth and development and to address questions in plant physiology, biochemistry, cell biology, and pathology.

Genetic and epigenetic factors determine leaf shape and structure

In angiosperms, leaf organs of single plants may not be uniform in shape, structure or function. The photosynthetically active foliage leaves often vary in size and shape according to the developmental age of the plant, as seen in *Arabidopsis*. As a result, an adult plant shows different types of leaves, a phenomenon called heteroblasty. The water plant *Ranunculus peltatus* develops different leaf shapes depending on submerged or floating conditions, so-called heterophylly. Leaves are sometimes modified to fulfill specific functions: bud scales, floral bracts and sepals are modified to protect vegetative buds, inflorescences, and flower buds, respectively; petals are modified leaves adapted to attract insects to flowers to aid pollination; cotyledons are converted to storage organs to sustain the growth of the seedling.

Leaves have evolved towards a shape with a big external surface and an inner cellular structure that allow efficient light capture and photosynthesis. Between families and even within a single plant family, the heritable genetic variation of leaf shape is enormous. The type of leaf, simple or compound, the leaf shape such as serrate, lanceolate, lobed or pinnate, and the pattern of venation is specific for each species and genetically defined, and has been used as a taxonomic criterion to classify species. Evolution is a driving force for this genetic diversity.

Heritable adaptations in leaf anatomy occurred in response to drought in xerophytes (desert plants) and to submerged conditions in hydrophytes (water plants).

From the molecular viewpoint this genetic variation can be explained in several ways. One could postulate that each species has the same set of genes and that leaf diversity is due to differences in gene expression. It is indeed well documented that changing the expression of even a single gene by mutation can dramatically alter leaf shape. For example, the *unifoliata* mutation in pea turns the normal compound leaf into a simple leaf (Marx, 1987).

Furthermore, the copy number of a gene may account for gene dosage effects. We have demonstrated that leaf size and shape in the *Arabidopsis pfl* mutant is changed due to the inactivation of one of three *RPS18* genes, important for translation initiation (Table 1; Van Lijsebettens *et al.*, 1994). Another hypothesis could have been that each species has a different set of genes the action of which could bring about leaf shape and structure in an independent way. However, the synteny of genetic maps between species of different plant families makes this hypothesis less plausible. Nevertheless, a molecular-genetic approach to investigate leaf formation will give answers to these questions.

Finally, there are non-heritable or epigenetic sources of variation in leaf size and shape in response to environmental conditions. Leaves enlarge and become darker green in shade whereas intense light induces small leaves with thicker wax protection, touch stimuli reduce leaf size. This flexibility in the leaf developmental programme is essential for rapid adaptation to a changing environment. The new phenotype is simply maintained for the life of the plant and not inherited.

Leaf ontogeny: a matter of genes?

Leaves are determinate structures that are formed as lateral outgrowths of the shoot apical meristem (Figure 1A) by a different rate and polarity of cell division between the outer (L1) and the inner (L2 and L3) layers. The upper and lower epidermis are derived from the L1 layer, the L2 and L3 layers contribute to the inner tissues. Procambial tissue develops from the base of the leaf primordium. Within the leaf primordium, patterns of cell division, cell expansion and differentiation ultimately lead to the bilateral asymmetric structure of the expanded leaf (Figure 1B).

Cell division follows a gradient from base to the tip and from the middle towards the margins of the developing leaf so that the "oldest" parts of a leaf are at the tip and the margins. These parts contain also the most expanded tissues since cell expansion is initiated at the lower (abaxial) side and proceeds towards the upper (adaxial) side and from the tip towards the bottom. This expansion creates the intercellular spaces within the spongy mesophyll. The palisade mesophyll is the last tissue to expand resulting in an increase in thickness, late in leaf development. During expansion, patterns of differentiation create a venation network, stomatal distribution and trichome positioning typical for every species. Subsequent leaves have a precisely determined position relative to one another, this pattern or phyllotaxis is laid down in the shoot apical meristem.

The phyllotaxis, architecture and shape of the leaves within a plant results from a cascade of coordinated steps, controlled by gene products of key regulatory genes. Subtle interactions between gene products, biochemical gradients and mechanical constraints superimposed onto the growing supracellular structure define the final size and shape of the mature leaf.

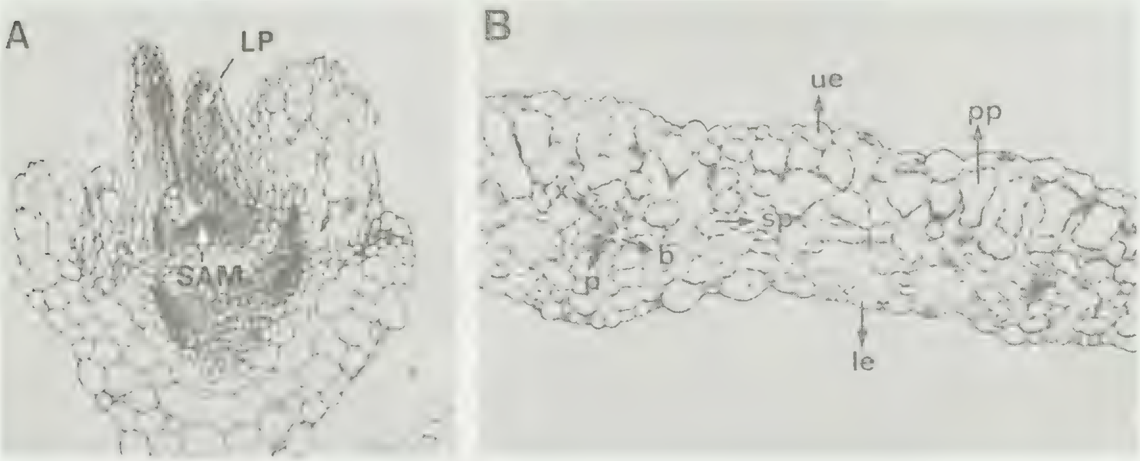

Figure 1 Leaf formation.

(A) Longitudinal section through a 12-day-old *Arabidopsis* seedling. LP, leaf primordium; SAM, shoot apical meristem. (B) Transverse section through an expanded *Arabidopsis* first leaf. b, bundle sheath, le, lower epidermis; p, phloem; pp, palisade parenchyma, sp, spongy parenchyma; x, xylem; ue, upper epidermis.

Insights into developmental processes in animal and plant systems have been obtained from mutational analyses, correlated with cloning and molecular analysis of the respective genes. In plants, genes that control steps in flower development and embryogenesis have been identified and studied. Mutational analysis has identified some of the genes that control steps in leaf morphogenesis. The cloning of these genes and the characterization of their corresponding proteins will greatly advance our understanding of leaf development.

Genetic loci controlling leaf formation: an overview

Upon mutagenesis, the DNA composition of the genome is changed: radiation induces base pair deletions while chemical mutagens, such as ethyl methane sulfonate, induce base pair substitutions. Natural mutagens in the form of mobile DNA elements or transposons are present in most of the plant genomes; examples are the *Activator/Dissociator* (*Ac/Ds*) system in maize and the *Tam3* transposon in *Antirrhinum*. These DNA entities maintain mobility by excising from their original insertion position to another site in the genome, generation after generation. Several transposons, including the maize *Ac/Ds* system, have been studied in detail and have been modified for use as insertion mutagens in heterologous species.

Another source of genetic modification, in plants, is the DNA transfer from the plant-pathogenic bacterium, *Agrobacterium tumefaciens*, to the genomes of a wide range of species upon infection. This transferred DNA or T-DNA has been adapted to be used as a vector for the introduction of foreign DNA into plants, it has also been exploited for the mutagenesis of the plant genome because of its random integration pattern.

Where the DNA mutations, induced by one of the described mutagens, occur within genes, gene expression may be blocked or changed resulting in the absence or alteration of the gene product. Mutations at single genes may result in a visible or measurable phenotypic change that becomes apparent in the mutated plant when dominant and in the next generation when recessive. The study of the defects in mutant plants provides information on the functional role of the respective gene product in the normal developmental process.

A considerable number of leaf mutants has been described in a range of plant species that identify genes important for specific processes during leaf morphogenesis. These are mentioned below.

- Homeotic mutants have leaf organs transformed into other organs or into modified leaf organs that are normally formed at other positions in the plant. In pea, the tendrils at the tip of the compound leaf are converted into leaflets in *the tendril-less* mutant, all leaflets are converted to tendrils in the *afila* mutant, while the *unifoliata* mutant has simple leaves due to the fusion of the leaflets of the compound leaf (Marx, 1987). In the *Arabidopsis leafy cotyledon* mutant, cotyledons are transformed into first leaves (Meinke, 1992), while in *extra cotyledon* the primary leaves are transformed into cotyledons (Telfer and Poethig, 1994).
- Heterochronic mutants have a shift in timing of their developmental programme. In maize, subsequent leaves of *teopod* mutants retain juvenile characteristics in contrast to wild type with an earlier switch to the adult phase (Dudley and Poethig, 1991).
- Cell identity mutants, such as *knotted* and *rough sheath* in maize, have localized zones of dedifferentiation and cell division at specific sites of the leaf lamina (Table 1; Sinha and Hake, 1990). Cell division activity is reduced in *pointed first leaves* (*pfl*) and *narrow leaf1* in *Arabidopsis* (Table 1, Van Lijsebettens *et al.*, 1994). The orientation of cell division is changed in the *lam* and *fat* mutant of *Nicotiana sylvestris* (McHale, 1993).
- Some mutants are affected in leaf and flower development such as *tousled* and *lopped1* in *Arabidopsis*, probably due to a defect in signal transduction (Table 1, Roe *et al.*, 1993; Carland and McHale, 1996).
- Cell elongation mutants, such as *angustifolia* and *rotundifolia* in *Arabidopsis* (Tsuge *et al.*, 1996), are defective in polar and non-polar cell expansion, respectively.
- Mutants in dorsoventral patterning have a conversion of dorsoventral asymmetry to radial symmetry with palisade parenchyma missing. Examples are the *phantastica* mutant in *Antirrhinum* (Waites and Hudson, 1995) and the *strongarm* mutant in *Arabidopsis* (J. Clarke and M. Van Lijsebettens, unpublished results). Epidermal pattern mutants with an altered density and/or distribution of the stomata have been isolated (T. Altmann, unpublished results) or with an altered pattern of trichomes (Hhlskamp *et al.*, 1994).
- Mutants defective in chloroplast differentiation lack proper palisade parenchyma (Table 1; Reiter *et al.*, 1994; Chatterjee *et al.*, 1996; Keddie *et al.*, 1996). Mutants defective in chloroplast division have either a few giant chloroplasts or a lot of small chloroplasts (Pyke and Leech, 1994).

A few genes with a role in leaf development have been cloned and are listed in Table 1. These genes have all been identified by mutation. Most of them have been cloned from alleles tagged by transposable elements in the homologous species (*KN1*, *PHAN* and *DAG*) or by their successful use in the heterologous species tomato (*DCL*) and *Arabidopsis* (*NRL1*), or by the *Agrobacterium* T-DNA in *Arabidopsis* (*PFL*, *GL1*, *GL2*, *TSL* and *PAC*).

Table 1
Genes identified by leaf mutants

Gene	Description	Process	Species	Cloning	Protein	Reference
KN1	knotted1	cell identity	Maize	*Ac/Ds*	homeobox type*	Vollbrecht et al. (1991)
RS1	rough sheath1	cell identity	Maize	*Mu*/DNA homology	homeobox type*	Schneeberger et al. (1995)
PFL	pointed first leaves	meristem activity	*Arabidopsis*	T-DNA	ribosomal protein S18	Van Lijsebettens *et al.*, (1994)
NRL1	narrow leaves1	meristem activity	*Arabidopsis*	*Ac/Ds*	homologue in yeast	M. Van Lijsebettens
PHAN	phantastica	dorsoventral patterning	*Antirrhinum*	*Tam3*	myb type*	A. Hudson
GL1	glabra1	trichome initiation	*Arabidopsis*	T-DNA	myb type*	Oppenheimer *et al.*, (1991)
GL2	glabra2	trichome development	*Arabidopsis*	T-DNA	homeobox type*	Rerie *et al.*, (1994)
TSL	tousled	signal transduction	*Arabidopsis*	T-DNA	kinase	Roe *et al.*, (1993)
PAC	pale cress	palisade differentiation	*Arabidopsis*	T-DNA	novel protein	Reiter *et al.*, (1994)
DAG	differentiation and greening	palisade development	*Antirrhinum*	*Tam3*	novel plastid protein	Chatterjee *et al.*, (1996)
DCL	defective chloroplasts and leaves mutable	chloroplast differentiation	Tomato	*Ac/Ds*	novel plastid protein	Keddie *et al.*, (1996)

Ac/Ds, *Mu* (*Mutator*), and *Tam3*, transposons; T-DNA, transferred DNA of *Agrobacterium tumefaciens*; *, regulatory protein.

Arabidopsis thaliana as a model system

The genetic interactions between the above-mentioned leaf mutations cannot be studied since they are obtained in different species and hence information from mutational analyses is not fully exploited. Concentration of molecular-genetic efforts on just a few model species will allow us to build genetic models for leaf morphogenesis. *Arabidopsis* (family *Brassicaceae*) is ideally suited for molecular-genetic studies due to its minimal genomic DNA content of 100 megabase, approximately five- to tenfold smaller than the genomes of important crops, such as rice, tomato, and *Brassica*, and more than 20-fold smaller than the maize and wheat genomes. Few repetitive sequences and small gene families account for technical and biological simplicity (Goodman *et al.*, 1995). Due to its small size it can be grown at high density (100 plants per 0.5 m^2) without seed contamination, and the short life cycle and the high seed production by self-fertilization make it amenable to laboratory-scale genetic experiments. The plant is easy to transform by tissue culture (Valvekens *et al.*, 1988) or *in planta* (Bechtold *et al.*, 1993) methods that allow the construction of transgenic plants without limitations.

In 1990, the "Multinational Coordinated *Arabidopsis thaliana* Genome Research Project" was initiated, supported by the National Science Foundation (U.S.A.) and steered by an international board of scientists. The aim was to promote *Arabidopsis* as a model for plants, in analogy to other models such as *Escherichia coli*, *Drosophila melanogaster* and *Caenorhabditis elegans* for other phyla. Within six years of time, functional DNA and seed stock centers were raised, one in Ohio (ABSRC, U.S.A.) and one in Nottingham (NASC, U.K.), an *Arabidopsis* database (aatdB) and an electronic newsletter were made available through Internet and yearly meetings are held to facilitate exchange and communication.

In Europe, an *Arabidopsis* genome sequencing project was initiated, followed by one in the U.S.A., with the aim of finishing one fifth of the genome in 1998, and the whole genome in the year 2004. The biological knowledge acquired from molecular-genetic studies on this model plant will be extended to a wide range of crop plants.

Molecular-genetic approach to study leaf development

In *Arabidopsis* a systematic genetic dissection of leaf morphogenesis has become a research topic in several laboratories. As yet, only a few gene loci have been located onto the genetic or molecular maps, but the number of mutants being analyzed is rapidly increasing. In our research group, mutational analysis is used to identify genes involved in leaf lamina formation. *Arabidopsis* populations mutagenized with either chemicals, the *Agrobacterium* T-DNA or the maize transposon system *Ac Ds* have been obtained and are being screened for altered lamina formation in the expanded first leaf. Several mutant classes have been obtained by single gene mutations of which some representatives are shown in Figure 2: the *pfl* (*pointed first leaves*) and *nrl1* (*narrow leaves1*) mutant with slightly or severely reduced width of the leaf lamina, respectively; the *sta* (*strongarm*) mutant with a serrate leaf lamina and a partially detached midvein; and the *trn1* (*tornado*) mutant with irregular lamina of which parts are missing and with a twisted petiole.

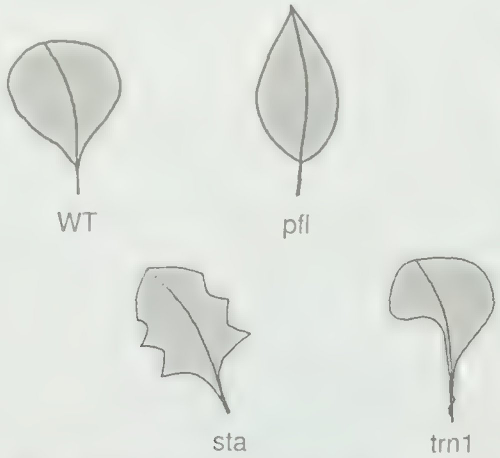

Figure 2. Arabidopsis *leaf mutants obtained by single gene mutations.* WT, wild type; *pfl, pointed first leaves; nrl1, narrow leaf1; sta, strongarm; trn1, tornado1.* Only midvein is represented.

A detailed morphological and anatomical analysis is being conducted on these mutants to determine whether all cell types are present, the cell number and/or size have changed, and whether the first defects are present in the leaf primordium, or occur later, during leaf maturation. Crosses are performed with mutants of similar phenotype and F1 progeny analyzed to determine whether the mutation is allelic to an existing mutant or represents a new genetic locus.

Where two different loci are mutated, the F2 generation will contain double mutants in which genetic interactions between the two loci can be examined. F2 and F3 analyses of a cross to wild type or to a tester line in another ecotype allows the determination of the map position of the locus relative to visible, selectable or molecular markers (Koornneef, 1990; Konieczny and Ausubel, 1993; Bell and Ecker, 1994; Van Lijsebettens *et al.*, 1996). Crosses to wild type are made to determine the linkage between the mutation and the T-DNA or Ds element, for mutants arising from T-DNA/*Ds* populations. No recombination between mutation and insertion elements indicates that the mutation has been tagged. These data are combined with molecular information obtained from cloning, sequencing and expression analysis of the gene to determine gene function.

We have developed several cloning strategies for isolation of genes identified by mutation. Where the mutated gene loci result from an insertion of a T-DNA or *Ds*, the known DNA sequence of these elements serves to isolate plant DNA sequences flanking the element by *in vitro* DNA amplification, so-called polymerase chain reaction (PCR). A thermostable DNA polymerase synthesizes a complementary DNA strand of a specific sequence in the genome due to the addition of short DNA stretches (primers) complementary to that sequence. Subsequent cycles of DNA denaturation, primer annealing and DNA synthesis result in the amplification of one specific DNA fragment out of the whole genome which is further analyzed. Figure 3 shows the specific amplification of T-DNA-flanking plant DNA in the T-DNA induced *pfl* mutant by inverse PCR (Vanderhaeghen *et al.*, 1992; Van Lijsebettens *et al.*, 1994). This method was also used to clone the Ds-flanking plant DNA in the *Ds*-induced *nrl1* mutant (unpublished data).

Mutant gene loci induced by chemicals have to be mapped relative to molecular and genetic markers in order to clone them by map based cloning techniques (chromosome walking). These methods rely heavily on genetic mapping using other ecotypes in which a proportion of the DNA sequence is divergent or polymorphic. This polymorphism is exploited to fine map the locus within a small genetic interval relative to molecular markers. These markers serve to identify a series of overlapping DNA clones containing the locus. Amplified fragment length polymorphism (AFLP) is a PCR based technique for the amplification of a subset of ecotype specific DNA fragments (Vos *et al.*, 1995). We have adopted the recently developed AFLP technique to create 17 new molecular markers within a 3 centiMorgan region around the *TRN1* locus.

Four of these markers cosegregate with the *trn1* mutation and allowed us to isolate one large DNA clone spanning the locus, illustrated in Figure 4 (Cnops *et al.*, 1996). This DNA fragment was subdivided into small fragments in order to isolate the *TRN1* gene.

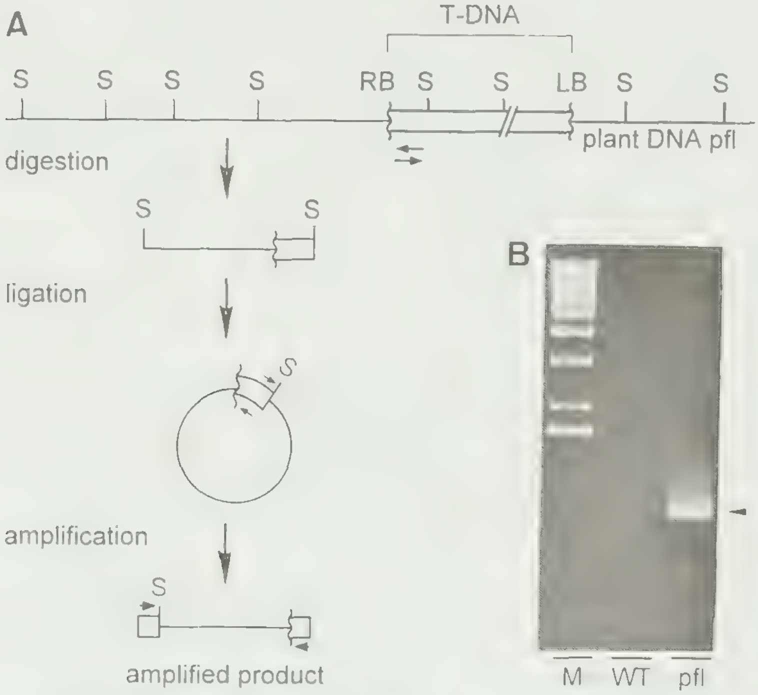

Figure 3 C *Inverse PCR amplification of plant DNA flanking the T-DNA in the* pfl *mutant.*
(A) The plant genome is cut with a restriction enzyme (S, *Sau3*A) to create a pool of DNA fragments; these fragments are enzymatically circularized; short DNA stretches (primers, indicated by arrows) complementary to the T-DNA border are added, and PCR amplification is performed. LB, left border; RB, right border; T-DNA, transferred DNA from *Agrobacterium tumefaciens*. (B) Result of this PCR reaction on wild type (WT) and *pfl* genomic DNA after gel electrophoresis. Amplified fragment is indicated by arrowhead. M is a DNA size ladder.

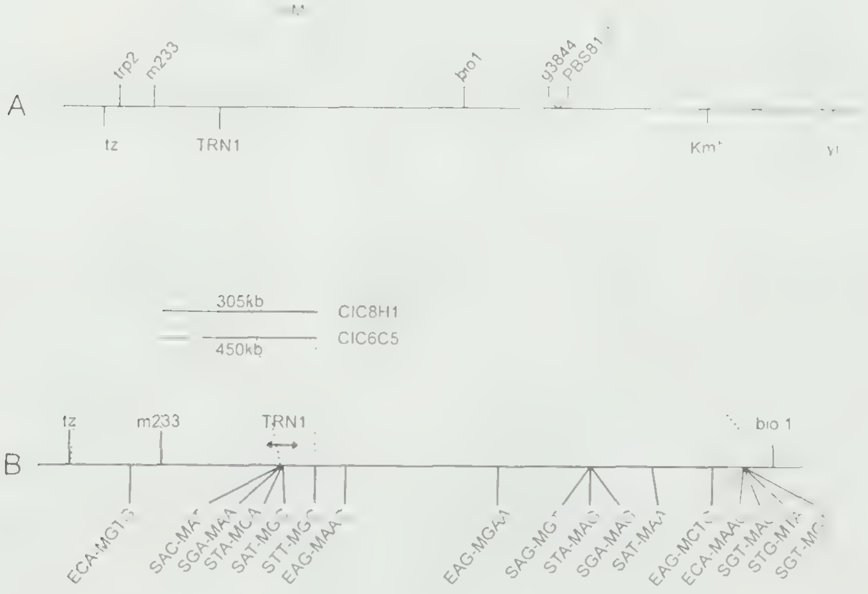

Figure 4 C *High resolution map of the* TRN1 *locus.*
(A) Part of chromosome 5 of *Arabidopsis* (top of the chromosome to the left, bottom to the right). Existing molecular markers are indicated at the upper side of the chromosome, genetic markers are at the lower side. (B) Enlargement of 3-centiMorgan region between the *tz* and bio1 marker. Newly-generated AFLP markers, indicated with code, are at the lower side of the chromosome. The CIC8H1 and CIC6C5 are big DNA clones that span the *TRN1* region. They are used to isolate the *TRN1* gene.

Once the gene has been cloned and sequenced, information on its function will be obtained if homology is found with known proteins from other organisms. Analysis of the deduced amino acid sequence for functional protein domains will indicate important enzymatic or structural features of the protein. Enzymatic assays will confirm the putative activities. The temporal and spatial expression of the respective gene has to be determined and the protein localized at the ultrastructural level.

Merging all the information obtained from these analyses will provide insight into the functional role of the protein in leaf development and will help to elucidate the complex nature of how the construction of the leaf is being realized.

Genes controlling lamina formation

One of the mutant classes we are studying identifies genes that act on lamina formation in a quantitative way, meaning that the reduction in lamina width is due to a reduction in cell number, but that all cell types are formed. With this class of mutants we would like to address the question whether the polarity of cell division within the shoot apical meristem is responsible for lamina formation or whether lamina formation merely depends on the existence and activity of a marginal meristem within the leaf primordium. Two tagged mutations of this type have been studied so far. The *pfl* mutation (Figure 2) has a moderately reduced width of the leaf lamina resulting in a 20% reduction in the fresh weight of fully grown rosettes. The respective gene, *RPS18A*, codes for the ribosomal protein gene S18, a homologue of the *E. coli* S13 protein known to interact with the translation initiation complex. The *RPS18A* gene is predominantly expressed in meristematic tissues. Null mutation at this gene is not lethal because of the overlapping expression of two additional copies of the gene family (Van Lijsebettens *et al.*, 1994).

The complex regulation of this gene family is being analyzed in more detail. The *nrl1* mutation (Figure 2) has an extremely narrow leaf blade, with normal length. This phenotype is already apparent at the primordium stage and is due to a reduction in cell number and size in the width direction. The *NRL1* gene encodes a protein homologous to a yeast protein with a putative role in the cell cycle control. However, its exact role in lamina formation remains to be resolved.

A second class of mutants, called *tornado* (Figure 2), has a completely deteriorated leaf lamina with parts of the blade missing and a defective vascular differentiation. They are severe dwarfs with twisted growth of all organs and reduced apical dominance. Several phenotypic aspects of the Trn mutant phenotype suggest that hormone action might be affected. Plant hormones are known to play an important role in a variety of developmental processes such as cell division and expansion, apical dominance, vascular differentiation, lateral root formation and tropisms. The genetic control of hormone biosynthesis, transport and perception is poorly understood, and few of the biosynthetic enzymes are known in plants. Alternatively, some aspects of the Trn phenotype, such as the twisting might be explained by a defect in the cell wall or cytoskeleton composition or arrangement. The DNA sequence of the respective gene will help to elucidate the role of the *TRN1* gene product in plant development.

Conclusion

Over the last few years, the tools have been designed to make the *Arabidopsis* genome accessible for gene cloning. Efficient transformation methods have resulted in the creation of large transgenic populations containing tagged alleles of genes of interest. These tools allow us to investigate problems in plant developmental biology. It is of utmost importance to focus on those mutations that will provide information on critical steps in developmental processes. Leaf development is virtually a blank field in which many interesting questions remain to be answered. The molecular mechanism determining meristem activity and determination, pattern formation in cellular and tissue differentiation, shape, size and the number of leaves and position along the axis have just started to be resolved. Knowledge will accumulate because efforts are being concentrated on one particular species; this will lead to models explaining the regulatory mechanisms and the genetic interactions underlaying leaf morphogenesis. One potential application from this research for agriculture or horticulture is expected to be genetic manipulation of leaf architecture and the creation of new varieties. Leaf anatomy, shape or positioning and orientation along the axis, or timing of leaf formation could be adapted to improve growth of crops or to adapt them to adverse conditions. Fundamental research is invaluable in order to obtain the basic knowledge and to collect a set of genes and promoters with specific activity during leaf development.

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Biophysical aspects of plant cell expansion: effect of the environment

Gail Taylor

School of Biological Sciences, University of Sussex, Falmer, Brighton, BN1 9QT, UK.
Tel: 44 1273 678872, Fax: 44 1273 678433, Email: g.taylor@sussex.ac.uk.

Introduction

Plant morphogenesis depends upon the production of plant cells through the plant cell cycle, the expansion of cells and their later differentiation. Understanding this co-ordinated development is fundamental to many areas of plant developmental biology (Green, 1994). An important aspect of this developmental sequence is the control of plant cell expansion; this is because the extent, timing and direction of cell expansion may have a significant effect on the development of organ and whole-plant form. In this short review, I will report the latest research in the area of plant cell biophysics and biochemistry, relevant to our understanding of plant cell growth. I will then identify how several important environmental variables influence plant growth, in the hope that this is of relevance to all involved in plant morphogenesis and the use of tissue culture systems.

The expanding plant cell: cell wall and cell water relations

Biophysical aspects of plant cell elongation

The increase in plant cell volume which occurs in many growing systems is phenomenal and contributes significantly to the shape and size of plant organs such as leaves and roots (Tomos, 1985). This volumetric increase in size is driven by the simultaneous uptake of water, extension of the cell walls and accumulation of solids. Although our knowledge of these processes is far from complete, work in recent years has illustrated the importance of cell wall processes in determining the rate of expansion of plant cells (Pritchard and Tomos, 1993). Enlargement is initiated when the wall is loosened by metabolic events and a turgor-driven extension follows. The cell wall has a central role in that no increase in size can occur without the irreversible extension of the wall. The term coefficient of cell wall extensibility (ϕ) was defined by Lockhart (1965). Cells which are more extensible generally expand more rapidly. Lockhart proposed that growth or strain rate could be defined by the following relationship:

$$\textbf{Growth (strain rate)} = \phi(P - Y)$$ [1]

where P is cell turgor pressure, measured in units of pressure (MPa) and Y is the yield turgor of the cell, also measured in MPa, whilst ϕ is measured as $MPa^{-1}\ h^{-1}$.

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[1] The term coefficient of cell wall extensibility ϕ, may also be given the symbol m, or refereed to as WEx (cell wall extensibility) or % Plasticity (irreversible extension of the cell wall). These terms, although qualitatively similar, differ quantitatively. See Taiz (1984) for a full explanation.

Lockhart was the first to realise that growth was initiated by a relaxation of 'wall stress', a process extremely difficult to quantify in growing plant systems, but often considered as equivalent to the 'turgor' within the cell, which can now be measured directly by the insertion of a miniaturised pressure probe into individual cells (Tomos and Pritchard, 1993) As extension occurs, turgor pressure initially decreases and creates a lowered water potential (Ψw) in the cell. The lowered water potential in the cell causes water to enter and occupy the new spaces created by the extension of the cell walls. Solute accumulation maintains the osmotic potential necessary to generate P and Ψw During steady growth these processes all occur simultaneously Although cell expansion is therefore a partial function of turgor pressure, it does not necessarily follow that this is the driving force for growth. Indeed, many recent papers have argued that the control point for cell expansion is actually cell wall relaxation and loosening In the Lockhart equation the yielding properties of the cell wall are defined by both ϕ and Y, yielding ability goes up as extensibility goes up and Y goes down. Evaluating the role of ϕ and Y in controlling expansion rate has been the subject of numerous recent reports. Some of these will be described later, but here we present the overriding evidence to show that the cell wall holds the key to understanding how plant cell expansion is controlled.

Cell wall biochemistry and biophysics - the control point for cell expansion?

The theory of initiation of cell expansion followed by wall relaxation was confirmed with the recent development of practical techniques for measuring wall relaxation (Cosgrove, 1993) These relaxation methods showed that when growing cells are constrained to a constant size, turgor and wall stress decreased, as expected, in a time-dependent manner - quickly for rapidly expanding cells and more slowly for slower growing cells. An early experiment by Cosgrove *et al.*, (1984) illustrated that this 'wall relaxation' could be accelerated by the treatment of stem tissue with IAA and quantified using either psychrometers or a pressure probe Interestingly, in such relaxing cells, turgor declined to the yield turgor Y, providing a useful technique for the assessment of this cell characteristic These observations all contradict the alternative theory which suggests that cell expansion begins with the osmotic uptake of water (uptake of solutes) and elastic expansion of the cell wall, followed secondarily by a breakdown and relaxation of the wall to reduce turgor and wall stress back to initial conditions Cosgrove (1993) argued that with such a mechanism, turgor would remain high, or even increase, when cells were constrained to a constant size, an observation rarely made This strongly suggests that the uptake of water is a process of secondary importance in the *control* of cell expansion, although much controversy still surrounds this idea.

For example, Boyer (1968), first suggested that small changes in leaf water potential could have a large and significant effect on leaf growth More recently, the concept that water influx may limit the growth of expanding cells has developed following demonstrations that 'water potential gradients' do exist in non-transpiring, growing stems (Nonami and Boyer, 1993) Despite these observations, however, the vast majority of evidence, as presented below, suggests that the cell wall has a pivotal role in the control of cell expansion If we believe this, then our next task is to understand the process of cell wall relaxation and extensibility Here, the structure and biochemistry of the cell wall has to be considered.

The plant cell wall consists of co-extensive networks of complex polysaccharides and structural proteins. In dicotyledonous plants, the major load bearing network consists of a skeleton of microfibrils (bundles of several cellulose polymers) interlaced by polymers of xyloglucan (Carpita and Garbeaut, 1993). The cross-linking xyloglucans are the major tension-bearing components in the longitudinal axis of an extending cell and recent research has suggested that this group of hemicelluloses may play a key role in controlling the process of cell wall relaxation (Fry *et al.*, 1992. Passioura and Fry, 1992).

Xyloglucan is a hemicellulosic polysaccharide that makes up approximately 20-25 % dry weight of the primary cell wall in dicotyledonous species. Xyloglucan chains are thought to 'tether' adjacent cellulose microfibrils and hence limit cell expansion. Some of these tethers are now thought to be load-bearing or 'taught' and it is the cutting of such tethers that allows the cell wall to extend. Such an extension would ultimately lead to the thinning a degradation of the cell wall and this is exemplified by the action of the enzyme cellulase on xyloglucan. Cellulase catalyses the irreversible cleavage of xyloglucan and is therefore considered unlikely as a true 'wall loosening' enzyme.

In contrast the enzyme 'xyloglucan endo-transglycosylase' (XET), both cuts and re-forms xyloglucan chains, allowing cell wall loosening whilst maintaining the integrity of the wall (Fry *et al.*, 1992). Since the initial discovery of XET as a wall loosening enzyme, several studies have demonstrated that increased cell expansion may be correlated with enhanced XET activity, for example in lettuce hypocotyls and cultured spinach cells (Potter and Fry, 1994), roots exposed to drought (Wu *et al.*, 1993) and leaves in elevated CO_2 (Ranasinghe and Taylor, 1996). Although the discovery of XET and its action on cell walls has added a new insight to our understanding of plant cell expansion, it may not be the only cell wall factor capable of controlling wall growth. For example, it has been difficult to show that acid-induced growth may be correlated to XET activity, whilst McQueen-Mason *et al.* (1993) have demonstrated the importance of two wall proteins which appear to act on the xyloglucan-cellulose network and are capable of causing extension growth in cucumber stems. These proteins have been termed expansins and have molecular masses of 29 and 30 kDa. They may act to loosen the wall by disrupting hydrogen bonding, although their significance has yet to be fully determined. In summary, much evidence exists to suggest that XET has a pivotal role in determining wall loosening and extension and that the tethering and untethering of load-bearing xyloglucan chains provides a realistic hypothesis, with supporting evidence to explain these phenomena, but that other proteins may also have a role in some plant systems.

Plant cell expansion and the environment

Plants are rarely subjected to optimal conditions for plant growth and environmental conditions may vary greatly over days and hours, during the production and expansion of plant cells. Understanding how the environment may influence plant cell expansion is fundamental to physiologists, developmental biologists and plant breeders alike. Here I consider how a number of key environmental variables may influence plant cell expansion. Throughout, the concepts of Lockhart (1965) are used as framework in an attempt to identify which aspects of expansion are most sensitive to the variable in question.

If we are able to quantify the effects of environment on cell processes such as wall loosening, water relations and yield turgor of cells, we are one step towards an improved understanding, also in selection and breeding for traits of importance in plant productivity.

Light and cell expansion

Both light quantity and light quality are known to influence plant cell expansion. Here, quantity effects will be restricted to a consideration of how PAR (photosynthetically active radiation, 400-700 nm) affects cell growth, whilst quality effects will consider both blue and red light PAR has a dramatic effect on the morphogenesis of leaves, and similarly on the growth and morphology of leaf cells. Plants grown at higher PARs are often characterised by thicker leaves, higher leaf mass per unit leaf area, higher concentrations of Calvin Cycle enzymes and electron transport carriers per unit leaf area (Sims and Percy, 1994) These characteristics lead to a higher photosynthetic capacity per unit leaf area in 'sun' as compared to 'shade' leaves Increased leaf thickness as facilitated by elongation of the palisade cells has been linked to a reduction in mesophyll resistance to CO_2 diffusion. Understanding these phenomena is therefore important.

Some advances were made in this area a few years ago when Van Volkenburgh and Cleland developed a system using leaves of *Phaseolus vulgaris* in which cell division was separated spatially from cell elongation, by the use of a red-light treatment (Van Volkenburgh and Cleland, 1979) This system was novel in that cell expansion is often difficult to study in dicotyledonous plants, since expansion and division occur simultaneously for much of the lamina growth phase For primary leaves of *Phaseolus*, exposure to high PAR light appears to be extremely important in promoting leaf cell expansion and growth Van Volkenburgh and Cleland, (1981) were able to show that this was not related to an effect on cell water relations, but rather to increased cell wall loosening and extension assessed from load-extension relationships using an instrument called an Instron. They were further able to show that increased cell wall extensibility was associated with an 'acid -growth' phenomenon similar to that observed following exposure of stems to IAA This mechanism does not, however appear to be of central importance for the expansion of leaf cells in all species.

Taylor and Davies (1985, 1986) illustrated clear species differences in cell wall properties and their role in the control of leaf growth For the sun-loving species *Betula pendula* cell wall extensibility and leaf growth were sensitive to PAR and increased growth was correlated with cell wall acidification, increased WEx and lower leaf turgor pressures (P). In contrast for the shade-tolerant *Acer pseudoplatanus*, PAR-induced leaf growth was absent as was any cell wall responses to increased PAR These data suggest that there may be a role for XET in explaining some of the species responses to light, observed to date. As yet, XET activity has only been quantified for *Phaseolus* leaves exposed to elevated CO_2 (see below), where maximum XET activity was correlated with the most rapid phase of leaf growth (Taylor *et al.*, 1994)

Temperature and cell expansion

In general, plant cell expansion is reduced as temperature is lowered. Few studies have considered the biophysical nature of reduced growth at low temperatures although leaves of *Lolium* are inhibited as temperature around the shoot meristem is reduced from 20 to 5°C. This is apparently independent of turgor, which is maintained irrespective of temperature (Tomos and Pritchard, 1994). A useful mutant to study temperature effects of leaf cell expansion was described recently by Schunmann *et al.*, (1994). The 'slender' mutant of barley is unusual in that leaf extension is often accelerated and this is most marked at a temperature of 5 °C. These authors have shown that slender elongation rates are the result of accelerated extension of individual cells and that this in turn occurs because of enhanced cell wall extensibility, since turgor pressure is equivalent in both wild type and mutant phenotypes. The Slender plants are characterised by at least five genes with altered patterns of expression although one of these, pcD31311 encodes for a shoot peroxidase. Peroxidases have been implicated in the control of cell wall hardening and may account for the wall phenotype observed in slender.

CO_2 and cell expansion

In future, the growth and development of plants will occur in a CO_2 environment considerably different from that today. Current concentrations of CO_2 (355 μmol mol^{-1}) are predicted to double by the end of the next century. A large amount of literature now exists on the likely effects of this change for whole plant physiology and productivity, but relatively few studies have concentrated on identifying how growth mechanisms will be affected by this rise in atmospheric CO_2. Work in our own laboratory has identified a number of post-photosynthetic growth processes which are sensitive to CO_2 supply in both leaves and roots. For leaves, area development is often stimulated in elevated CO_2.

We have observed this effect for a wide range of species, from native herbs to trees such as *Populus* (Ferris and Taylor, 1994; Gardner *et al.*, 1995). Interestingly, both the production and expansion of leaf cells may be promoted in elevated CO_2, but in our studies, large growth effects were only apparent when cell expansion was promoted. Once again, the cell wall appears to be critical in explaining this stimulation in growth which appears to occur because of increased cell wall extensibility. For *Plantago media*, a common native herb, LVDTs (linear transducers for following leaf growth non-destructively), reveal that elevated CO_2 promotes night-time rather than day-time growth, although the mechanism is similar with cell wall extensibility enhanced. Interestingly, species differences are apparent, since for a second native herb, *Anthyllis vulneraria*, growth is promoted in elevated CO_2 as a result of increased cell turgor pressure with no significant effect on cell wall properties.

These studies confirm the original ideas of Cosgrove on the role of the cell wall, suggesting that wall relaxation is the primary control point for expansion, but that exceptions may exist to this rule. Finally, we have also shown that the enzyme XET is also sensitive to the supply of CO_2 in the model bean leaf system, where activity was enhanced for leaves exposed to elevated CO_2 during the phase of cell expansion (Ranasinghe and Taylor, 1996). Carbon dioxide is an interesting treatment since cells may respond directly to the gas, or indirectly following photosynthetic fixation of carbon into sugars.

In non-photosynthesising tissues, it has been suggested that CO_2 may act on the ethylene formation pathway, although evidence for this effect is limited. For post-photosynthetic responses, root cells provide a useful system for study. This is because they consist of regular arrays of cells at different stages of development, beginning with the meristematic cells of the root tip and continuing through to the differentiated cells and the formation of vascular tissue. We have hypothesised that exposure of shoots to elevated CO_2 leads to an increase in the transport of photoassimilates to root cells. There are two effects of this additional carbon. Firstly, the rate of expansion of primary root cells is accelerated, although final cell size may be unaltered (Taylor *et al.*, 1994) and secondly, the formation of lateral branches is enhanced.

This latter effect is like you reflect an increase in cell cycle activity (Kinsman *et al.*, 1995) and will not be considered further here. The effect on primary root cell expansion is of interest since a considerable amount of research has been undertaken on the control of root cell expansion (Tomos and Pritchard, 1993). These authors and others, have highlighted the importance of the cell wall in explaining both the accelerating and decelerating phases of growth, which are readily separated in this linear growth system. Interestingly, very different growth rates may be maintained across these cells, but turgor pressures within the cells from tip to base areas of individual roots are remarkably similar. Pritchard and Tomos (1994) have suggested that the acceleration phase of root cell expansion may be explained by enhanced extensibility, whilst the deceleration phase results from an increase in Y, the yield turgor.

Water and cell expansion

Because water is intimately involved in the process of cell expansion, it seems likely that its removal would have a dramatic effect on cell growth and this is certainly found to be so. In the short term, root and leaf growth may both be extremely sensitive to water supply. For droughted plants, most recent research has considered the likely role of hydraulic versus chemical signals in regulating shoot physiology and growth. It now seems likely that abscisic acid acts as a root signal, moving from dehydrated root tips in the xylem stream, to growing shoots and leaves (Zhang and Davies, 1990).

Once in leaves, ABA may act to reduce stomatal conductance, but is also known to have a potent effect as a growth retardant. The implications of these findings are wide-ranging, suggesting that any cell exposed to dehydration may produce ABA, with concomitant physiological effects. For example, Wu *et al.* (1993) have shown that roots exposed to very mild dehydration may extend more rapidly and that this is accompanied by an increase in XET activity. In contrast, such a treatment usually results in a rapid decline in the rate of expansion of leaf cells, although the reason for this remains unclear. Even a few root tips exposed to dehydration may be adequate to trigger this response, with wide-ranging consequences for plant cells.

Conclusions

This short paper has described the biophysical and biochemical aspects of plant cell expansion thought to be important for control. Recent research suggests that the cell wall, rather than cell water relations, may be more important in controlling cell expansion, in a number of plant systems. Under such circumstances, rapid growth is often associated with *decreased* turgor pressure and yield turgor and an *increased* cell wall extensibility. The environment may affect the expansion rate of both leaf and root cells, but this paper has illustrated that often, the cell wall appears to be sensitive to changed conditions and it is through cell wall biochemistry and biophysics that cell expansion is altered following environmental perturbation. Future research should focus on processes which contribute to this cell wall control. The enzyme xyloglucan endo-transglycosylase (XET) has already been implicated and cDNAs are now available for several XETs (de Silva *et al.*, 1994).

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Genetics of flower development

Tamara Maes, Nancy Van de Steene, Marc Van Montagu and Tom Gerats

Laboratorium voor Genetica, Department of Genetics, Flanders Interuniversity Institute for Biotechnology (VIB), Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium;

Introduction

Goethe, inspired by his study of homeotic variation in plants, already formulated the hypothesis that the four types of floral organs are essentially modified leaves. As in those days, current models for flower development are based on the phenotypes of plants with aberrant flowers or inflorescences. The molecular genetic characterisation of the involved genes in *Arabidopsis thaliana* and *Antirrhinum majus*, and the comparative analysis of their homologues in a range of species is well under way. This review is mainly limited to the discussion of the analogies and the differences between three model systems: *Arabidopsis thaliana*, *Antirrhinum majus* and *Petunia hybrida*.

About flowers and inflorescences

In hermaphrodite angiosperm flowers one can distinguish four types of organs, that are often organised in concentric whorls. At the periphery of the flower the sepals can be found. The sepals have been proposed to be derived from the bracts, and serve a mainly protective function. The petals are the display equipment of the flower, they are often white or brightly coloured or exhibit patterns to attract and guide pollinators. In the centre of the flower the sexual reproduction organs are located, the stamens and a central gynoecium consisting of fused carpels that harbours the ovules. Flowers can be asymmetrical or symmetrical, in the latter case they are called *zygomorphic*, *bisymmetric* or *actinomorphic* depending on whether they exhibit one, two or more planes of symmetry (Weberling, 1989).

Flowers are organised in inflorescences. According to Troll, the inflorescence is a modified shoot system that serves for the formation of flowers (Troll, 1964). Inflorescences can be *determinate*, if they end in a terminal flower or *indeterminate*, if they do not. Branching within the inflorescence is called *racemose* if there is a clearly developed main axis that produces only lateral flowers, or *cymose*, when the branching occurs exclusively from the axils of the inflorescence leaves or prophylls (Weberling, 1989). While floral morphology has been described for thousands of species from an anatomical point of view, their molecular biology has been studied only in a handful of species.

Arabidopsis, *Antirrhinum* and *Petunia*: Model Systems for Molecular Genetics

Arabidopsis, *Antirrhinum* and *Petunia* each have their own advantages as model systems in molecular genetics.

Arabidopsis has a relatively small genome size, making map based cloning of genes a feasible approach. *Petunia* and *Arabidopsis* are susceptible to *Agrobacterium* mediated T-DNA transformation.

Libraries of T-DNA lines can be screened for plants with mutant phenotypes and the corresponding genes can be cloned relatively easy if they are caused by the T-DNA insertion. T-DNA transformations can also be used to introduce a gene under a strong promoter in order to study the overexpression phenotype.

In species like *Petunia*, where most of the flower development genes have been cloned by homology, the function of a gene can be analysed in plants where its expression has been reduced by anti-sense or by sense (a phenomenon called co-suppression) transgenes.

Transposable DNA elements may cause mutant phenotypes when inserted in the coding sequence or in an important regulatory region of a gene. Transposon tagging has been used in *Antirrhinum* and *Petunia* to isolate developmentally important genes. In case the gene but not its function is known, a powerful PCR based method can be used to screen populations for plants that contain an insertion in the gene of interest.

Short description of the development of the three model plant systems

After germination, the vegetative meristem of *Arabidopsis thaliana* produces a rozette of spirally positioned leaves, separated by short internodes. Upon floral induction the apical meristem changes fate and becomes an inflorescence meristem. The inflorescence meristem generates a small number of cauline leaves in the axils of which secondary inflorescences will arise. On the flanks of the inflorescence meristem, flower primordia arise. These develop into actinomorphic flowers, consisting of four concentric whorls of organs. The two outer circles are collectively called the perianth and contain four sepals and four petals, the third whorl contains six stamens, and the inner whorl two fused carpels.

The vegetative phyllotaxy of *Antirrhinum majus* is decussate, i.e. with opposite leaf pairs being generated at right angles along the axis. The transition of the apex from a vegetative to an inflorescence state is accompanied by a change in phyllotaxy. The inflorescence leaves or bracts are generated in a spiral along the axis. In the axils of the bracts, the flower primordia develop into zygomorphic flowers containing five sepals, five petals, five stamens and two fused carpels.

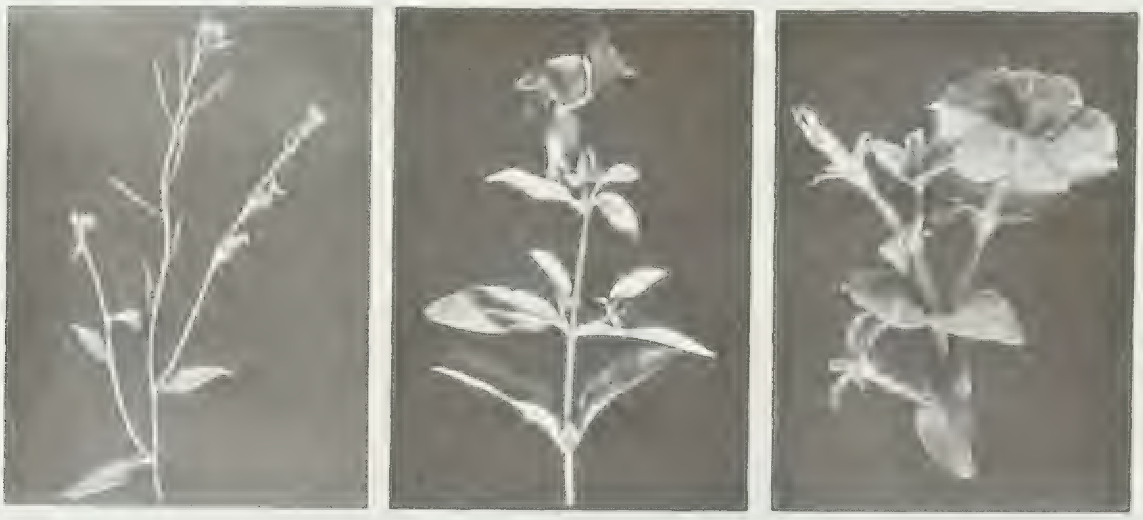


Figure 1: Wild type inflorescences of *Arabidopsis thaliana* (left), *Antirrhinum majus* (middle) and *Petunia* (right)

Both Arabidopsis and Antirrhinum are clear examples of racemic inflorescences. In Petunia hybrida vegetative growth is spiral. Upon floral induction, the inflorescence apex produces two inflorescence leaves (α,β) called prophylls under an angle of about 150 degrees. The apical meristem divides in two to form a floral meristem and an inflorescence meristem. The inflorescence meristem generates the next set of prophylls, and the developmental sequence described above is repeated. This inflorescence branching pattern lacks a clearly developed main axis and is termed cymose. *Petunia* flowers are zygomorphic, and consist of five sepals, five petals, five stamens, and two fused carpels (Turlier and Alabouvette, 1988).

The flowers of the three model species that will be discussed are presented in figure 1.

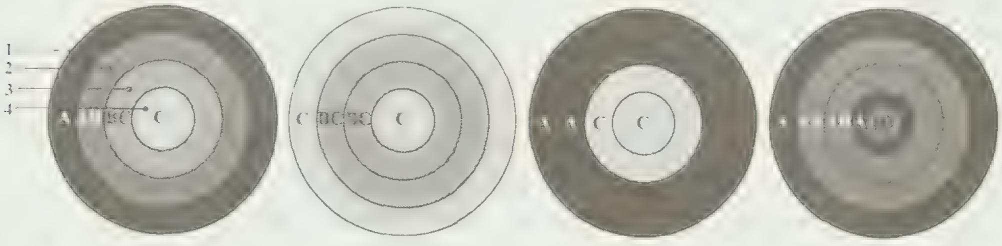
Determination of floral whorl identity

In homeotic mutants, one type of organ is replaced by another type of organ. Homeotic transformations can arise as a consequence of the loss of activity of an organ identity gene or alternatively as a result of a change in its spatiotemporal expression pattern. The organ identity genes are the initial inducers of the genes that will ultimately determine the morphology of the different organs. Genes that determine the expression pattern of the organ identity genes are called cadastral genes. The expression of the cadastral and the organ identity genes is superimposed on a pattern of organ primordia initiation. The genes that affect these processes in floral development and their interactions will be discussed below.

The ABC model for the determination of floral organ identity

Flower homeotic mutants have been isolated for many species, but they have been studied especially well for *Arabidopsis thaliana* and *Antirrhinum majus*. Mutants affecting the whorl identity in *Arabidopsis* and *Antirrhinum* can be assigned to three different classes. Each of these classes affects the identity of organs in two adjacent whorls of the flower. In class A mutants the sepals in whorl one are replaced by carpels, the petals in whorl two by stamens. Class B mutants have sepals and carpels instead of petals and stamens. In class C mutants the third whorl stamens are transformed into petals and the carpels into sepals. In addition, the determinancy of the flower of class C mutants is affected, i.e. the (mutant) flower pattern is repeated within the fourth (and normally last) whorl (figure 2).

The ABC model proposes that three functions (A,B and C) exist that determine the identity of a developing floral organ primordium in a combinatorial way (Bowman *et al.*,1991, Coen, 1991, Coen and Meyerowitz, 1991, Meyerowitz, *et al.*, 1991). Activity of A alone will lead to the formation of sepals in whorl one. In combination with B, petals will be formed in whorl two. The combination of B and C in the third whorl will result in stamen specification, and C alone is sufficient to develop carpels in the innermost whorl (figure 2). The mutant phenotypes of the class A and class C mutants can only be explained if their activities are mutually antagonistic, i.e. if loss of activity of one of these functions implies a gain of function of the other one. For example, in the absence of the A function, sepals and petals are replaced by carpels and stamens because the C function is now active also in the first and the second whorl. In plants that are mutant for both the A and the B function, only carpels are formed. Loss of both the B and C function results in formation of sepals in all whorls, and the determinancy of the flower is lost.



wild type class A mutant class B mutant class C mutant

Figure 2: The ABC Model For Floral Organ Identity Determination

The identity of the organs in the different whorls, represented by concentric circles, is determined by the overlapping activities of three classes of genes In the first whorl, activity of class A genes determines the formation of sepals (■). In the second whorl, the combination of the A and B class genes results in the formation of petals(▨). Activity of both the B and C function results in the formation of stamens (▒) in whorl three, and C alone is sufficient for the formation of carpels (□) in the fourth whorl. In class A mutants, C is expressed ectopically, which results in the formation of carpels in whorl one and stamens in whorl two. In class B mutants, petals in whorl two are transformed into sepals, and stamens in whorl three into carpels. In class C mutants, the A funotion is active also in the inner two whorls, which results in the formation of petals in the third and sepals in the fourth whorl In addition the determinancy of the flower is lost and the mutant pattern is repeated within the fourth whorl (After Bowman et al ,1991, Coen, 1991, Coen and Meyerowitz, 1991 and Meyerowitz et al ,1991)

These double mutant phenotypes show that the establishment of the B function is independent of A and C and that their respective mutant phenotypes are additive. The phenotype of the triple mutant combination was correctly predicted by the model and has proved Goethe (1790) right in his hypothesis after a time lapse of about 200 years: in the triple mutant, the floral organs remain organised in whorls but they look very much like vegetative leaves (Bowman et al, 1991).

In order to limit confusion, the organ identity genes that have been isolated from *Arabidopsis*, *Antirrhinum* and *Petunia* will be treated according to their function, rather than according to the species they belong to An overview of the homologous genes in the three different species is presented in table1.

A and C function genes

The *Arabidopsis Apetala2* (*Ap2*) gene is necessary for the normal development of sepals and petals In *ap2* mutants, carpels and stamens develop in the first and the second whorl (Komaki *et al.*, 1988, Bowman *et al.*, 1989, Kunst *et al.*, 1989) The C function gene *Agamous* (*Ag*) is expressed in the third and fourth whorl of wild type *Arabidopsis* flowers, and required for the development of stamens and carpels, that are replaced by petals and sepals in the *ag* mutant (Yanosfky *et al.*, 1990). In *ap2* mutants, *Ag* is also expressed in the first and the second whorl, showing that one of the functions of *Ap2* is to negatively regulate *Ag* in that part of the flower (Drews *et al.*, 1991). Transgenic *Arabidopsis* plants that overexpress *Ag* under control of the constitutive CaMV35S promoter have an *ap2*-like phenotype, which is consistent with the proposed antagonistic roles of *Ap2* and *Ag* (Mizukami and Ma, 1992).

Apart from its role in the development of the perianth in *Arabidopsis*, *Ap2* also has a function in the determination of floral meristem identity (see below) and in the development of the seedcoat *Ap2* is expressed throughout floral development (Jofuku *et al.* 1994), which raises the question of how *Ag* may be repressed by *Ap2* in the perianth, but not in the third and fourth whorl. Since *Ap2* expression itself is ubiquitous, other factors must be involved to determine the spatial specificity of the repression of *Ag*. This specificity might arise in many ways: the AP2 activity could be regulated by protein modification, the subcellular localisation of the protein might be controlled, or alternatively AP2 might be part of or induce the formation of a repression complex that includes (a) perianth specific factor(s).

Apetala1 (*Ap1*) is one of the genes that might play a role in this process. Single *ap1* mutants have flowers with cauline first whorl organs while new mutant flower shoots develop at the normal position of the petals (Irish and Sussex, 1990). Unlike *Ap2*, *Ap1* is expressed specifically in the outer two whorls of the wild type flower (Mandel and Yanofsky, 1995). Ectopic *Ag* expression can sometimes be seen in ap1 mutants (Weigel and Meyerowitz, 1993), supporting the idea that *Ap1* could help to specify *Ag* repression in the perianth.

In *Antirrhinum* and *Petunia*, mutants have been isolated that show homeotic transformations corresponding to a (partial) loss of the A function. The *ovulata* (*ovu*) phenotype in *Antirrhinum* is reminiscent of *ap2* in *Arabidopsis*, but rather than from a loss of A function it stems from a gain of C function caused by the insertion of a transposable element in a regulatory region of the *Plena* (*Ple*) gene (Bradley et al, 1993). The *fistulata* (*fis*) mutant in *Antirrhinum* and the *blind* (*bl*) mutant in *Petunia* have antheroids instead of petal lobes, while a weak carpeloidy may occur on blind sepals (Stubbe, 1966, Vallade et al, 1986, Tsuchimoto et al, 1993). Also, transgenic plants that express the *Petunia* C class gene *pMADS3* ectopically, phenocopy the *bl* mutant, suggesting that *Bl* might be a *Petunia Ap2* orthologue (Tsuchimoto et al, 1993). However, *Bl* does not coincide with one of the three *Ap2*-like genes in Petunia, and the *Ap2* copy that shows the highest homology with the *Arabidopsis* gene seems to be redundant in flower development, since loss of function mutants don't show a homeotic phenotype (Tamara Maes *et al.*, unpublished results). The redundancy might be explained if one of the other *Ap2*-like genes can substitute for its function, which will have to be analysed in the double or triple knock-outs. Alternatively, the *Ap2* homologues in *Petunia* might not be involved at all in the specification of the organ identity in the perianth.

B function genes

Deficiens (*DefA*) and *Globosa* (*Glo*) of *Antirrhinum* are expressed in the second and third whorls of the wild type flower (Sommer *et al.* 1990, Sommer *et al.*, 1992). Mutations in either *DefA* or *Glo* lead to a homeotic transformation of petals into sepals and of stamens into carpels. It was shown that the transcription of both *DefA* and *Glo* is downregulated in *defA* mutants that produce a mutant DEFA protein. Moreover, the DEFA and GLO gene products can form a heterodimer that binds to target elements located in the promotor of both genes *in vitro*. It was proposed that DEFA and GLO form a heterodimer that binds also *in vivo* to target elements in the promotors of both genes and thereby upregulates their initially low expression in the second and third whorl (Schwarz-Sommer *et al.*, 1992, Tröbner et al, 1992).

In contrast to *DefA*, their Petunia homologue *pMADS1* is redundant for stamen formation. The *green petals* mutant (*gp*) of *Petunia* lacks the *pMADS1* gene (van der Krol *et al.*, 1993). In the *gp* mutant, sepals develop instead of petals but the formation of stamens is not disturbed (Wiering *et al.*, 1979). Unlike in *Antirrhinum*, the expression of *FBP1* and *pMADS2*, the *Glo* homologues of *Petunia*, can be induced independently of *pMADS1* in the third whorl, which may account for the development of the anthers (Angenent *et al.*,1992, 1993, 1995).

The B function genes of *Arabidopsis*, *Apetala3* (*Ap3*) and *Pistillata* (*Pi*), are both necessary for the formation of petals and stamens (Jack *et al.*, 1992, Goto and Meyerowitz,1994). However, in transgenic plants that express *Ap3* ectopically in the whole flower, the endogenous *Pi* gene is also switched on in the fourth whorl, which is sufficient to transform the carpels into stamens (Jack *et al.*, 1994).

On the other hand, plants ectopically expressing *Pi* in all four whorls show a partial first whorl conversion of sepals into petals, because the endogenous *Ap3* is expressed also in the first whorl. In plants that express both *Ap3* and *Pi* ectopically both sepals and carpels are replaced by petals and anthers, respectively, which shows that the combined expression of *Ap3* and *Pi* is sufficient to provide the B function in any type of floral organ (Krizek and Meyerowitz, 1996).

Cadastral genes

Cadastral genes determine the spatial expression pattern of the organ identity genes. Some genes may have a dual function and can act both as a cadastral gene and an organ identity gene. The organ identity gene *Ap2*, for example, is also required for the repression of *Ag* expression in the outer two whorls of the flower Other genes have a purely cadastral function. Mutations in *Leunig* (*Lug*) can enhance the weak *ap2-1* mutant phenotype (Liu and Meyerowitz, 1995).

Flowers of *lug* single mutants have carpeloid, staminoid or petaloid sepals and exhibit antheroid petals or loss of petals Carpeloidy in sepals and antheroidy in petals can be explained by the ectopic expression of *Ag* in *lug* mutants *ap2 ag* double mutants form leaf-like organs in whorl one, organs intermediate between stamens and petals in whorls two and three, and another mutant flower in whorl four On the other hand, lug/ag double mutants make (narrow) sepals in whorl one, and (narrow) petals in whorl 2 and 3, after which the pattern is repeated in whorl 4 This illustrates that *Lug* is, unlike *Ap2*, not necessary for the determination of the identity of these organs and has a purely cadastral function Apart from *Ag*, also *Ap1*, *Ap3* and *Pi* have an altered expression pattern in lug mutants (Liu and Meyerowitz, 1995).

In the absence of a functional *Superman* (*Sup*) allele, extra stamens are formed at the expense of carpels in the fourth whorl (Bowman *et al.*, 1992, Schultz *et al.*, 1991). The ectopic carpels in the first whorl of *sup ap2* double mutants develop as in single *ap2* mutants, showing that *sup* is not necessary for carpel formation per se. The *ap3* or *pi* single mutants and the *sup ap3* or *sup pi* double mutants have the same phenotype (Bowman *et al.*, 1992, Schultz et al, 1991b). This suggests that in wild type, *Sup* is positively regulated by *Ap3*, or that *Sup* is necessary to repress *Ap3* and *Pi*. *Sup* is expressed adjacent to the boundary between the third and the fourth whorl There are two hypotheses to explain how *Sup* might function According to the first hypothesis, *Sup* is a cadastral gene that represses the expression of the B function genes *Ap3* and *Pi* in the fourth whorl The other theory is that *Sup* represses cell division in the cells at the boundary between the third and fourth whorl In the *sup* mutant, these cells proliferate and as a consequence extra stamens are formed at the expense of the meristem remaining for the carpel formation (Sakai *et al.*, 1995)

Initiation of floral organ primordia

Other genes like the *Clavata* (*Clv*) loci and *Perianthia* (*Pan*) in Arabidopsis and *Double1* (*Do1*) and *No Apical Meristem* (*NAM*) in *Petunia*, determine the sites and the number of floral organ primordia that will develop within a whorl (Clark *et al.*, 1993, 1995; van der Krol and Chua, 1993, Souer *et al.*, 1996).

Flowers of *clv1* and *clv3* plants can have increased numbers of organs in all whorls and can form extra whorls in addition *Arabidopsis pan* plants have pentamerous flowers with five sepals, five petals and five stamens instead of four sepals, four petals and six stamens (Running and Meyerowitz, 1996).

Arabidopsis	Antirrhinum	Petunia
Organ identity genes		
A function		
Ap2		*Ap2A,B,C ?*
Ap1	*Squa*	
	Fis	*Bl*
B function		
Ap3	*DefA*	*Gp (pMADS1)*
Pi	*Glo*	*FBP1*
		pMADS2
C function		
Ag	*Ple*	*FBP6/ pMADS3*
Cadastral genes		
Sup		
Lug		
Ap2		
Ag	*Ple*	*FBP6/ pMADS3*
Initiation of organ primordia		
Clv1, Clv3		
Pan		
		Do1
		NAM
Floral meristem identity genes		
Lfy	*Flo*	*Alf*
Ap1	*Squa*	
Cal		
Ap2		
Tfl1	*Cen*	
Intermediate between flower meristem and organ identity genes		
UFO	*Fim*	
Asymmetry genes		
	Cyc	

Table 1: Genes involved in flower development in *Arabidopsis, Antirrhinum* and *Petunia*.

The dominant *Do1* mutation in *Petunia* results in the formation of numerous extra petaloid or stamenoid organs or stamens in the inner whorls of the flower, which resembles the phenotype of *sup* mutants in *Arabidopsis*. While the *sup* phenotype disappears in a *pi* or *ap3* background, the *Do1* and *gp* or *bl* mutant phenotypes in Petunia are additive, therefore these genes act independently of each other (van der Krol and Chua, 1993).

The *NAM* gene of *Petunia* has a function in pattern formation in embryos and in flowers (Souer *et al.*, 1996). Instead of five, ten primordia develop in the second whorl of nam flowers and nam/gp double mutants, illustrating that *NAM* acts to determine the number and position of the primordia that are formed in whorl two. In situ hybridisation analysis with *NAM* has shown very nicely that it is expressed at the boundaries of meristems and primordia, before these start to develop as separate entities.

The different classes of mutants have been found to occur in all three (and other) model systems, which suggests that the majority of the key genes involved in floral organ determination has been discovered. While the different homologous genes play similar roles in the three different species, it is also clear that each species has its own particularities.

Determination of floral meristem identity

A meristem within an inflorescence can develop either as a secondary inflorescence shoot, or it can terminate and become a flower. The choice that is made between these two alternatives during the ontogeny of the inflorescence, will determine much of its final form. A number of genes has been isolated in *Antirrhinum* and *Arabidopsis* that have a function in the developmental switch between inflorescence and floral identity.

In Antirrhinum

Floricaula (*Flo*) was the first floral meristem identity gene to be isolated. In *flo* mutants the meristems in the axils of the bracts develop into new inflorescences instead of into flowers (Coen *et al.*, 1990).

In wild type *Antirrhinum* plants, *Flo* is transcribed in the floral meristem and in the adaxial region of the subtending bracts. *Squamosa* (*Squa*) is also involved in the transition from inflorescence to floral identity. In squa mutants, flowers are partially transformed into inflorescence shoots (Huijser *et al.*, 1992). In contrast, *Centroradialis* (*Cen*) is necessary to maintain the indeterminancy of the inflorescence axis (Coen, 1991).

Upon floral induction in cen mutants, *Flo* is induced (as in wild type) in the developing floral primordia and their subtending bracts. However, after 6 to 16 normal flowers have been initiated, *Flo* is also expressed in the inflorescence apex. This results in the formation of a terminal flower which is, unlike the normal axial flowers, radially symmetrical. Although *Flo* and *Cen* perform antagonistic functions in the determination of floral meristem identity, *Flo* is necessary for the early induction of *Cen* in the subapical region of the inflorescence. In *flo* mutants that produce a non-functional protein, *Cen* expression is not induced. As a consequence, *Flo* expression is not repressed and mutant *Flo* mRNA can be detected in the apex (Bradley *et al.*, 1996).

In Arabidopsis

Inactivation of the *Arabidopsis* homologue of *Flo*, *Leafy* (*Lfy*) results in a (partial) transformation of flowers into new inflorescence shoots (Schultz and Haughn, 1991a,Weigel *et al.*, 1992). In basal inflorescence shoots of *lfy* mutants the expression of the flower homeotic genes *Ap3* (B function) and *Ag* (C function), is not induced (Weigel and Meyerowitz, 1993). However, the formation of carpels at the end of the inflorescence shoots in the more apical shoots of *lfy* mutants shows that *Ag* can be induced independently of *Lfy* activity at a later stage in development. This alternative flower induction route is provided by the *Ap1* gene product (Bowman *et al.*, 1993). *ap1* Mutants have flowers with cauline first whorl organs and at the normal position of the petals a new mutant flower shoot develops (see above). The function of *Ap1* in the determination of floral meristem identity is illustrated by the complete conversion of flowers into inflorescence shoots in the *lfy ap1* double mutant combination (Bowman et al, 1993) .

The *Cauliflower* (*Cal*) gene seems redundant in flower development since *cal* mutants have a wild type phenotype. Mutations in *Cal* can greatly enhance the phenotype of weak *ap1* mutants. In double mutants where *cal* is combined with strong *ap1* alleles, the flower primordium undergoes multiple rounds of meristem production resulting in a "cauliflower" phenotype (Bowman *et al.*, 1993, Kempin *et al.*, 1994). The organ identity gene *Ap2* also has a minor function in the determination of the identity of the floral meristem, since *ap2* mutants can enhance the phenotype of *lfy* mutants (Huala and Sussex, 1992).

Overexpression of either *Lfy* or *Ap1* under control of the CaMV35S promotor is sufficient to confer floral identity to lateral shoots in *Arabidopsis* transgenics, which results in precocious flowering (Weigel and Nilsson, 1995, Mandel and Yanofsky, 1995). The capacity of *Lfy* to commit developing lateral meristems to flowering has also been applied successfully to induce early flowering in the tree aspen (Weigel and Nilsson, 1995).

While *Lfy* activity seems sufficient to commit floral identity to lateral shoots, *Terminal flower* (*Tfl1*) fulfils an antagonistic function and is required to maintain the inflorescence identity of the apex (Shannon and Meeks-Wagner, 1991). Consequently, *tfl1* mutants have a shortened vegetative phase, flower early and develop an inflorescence that ends prematurely with a terminal flower, and thus resemble *Lfy* overexpression plants. *Tfl1* is the orthologue of *Cen* in *Arabidopsis*. In *Antirrhinum*, expression of *Flo* precedes and is required for induction of *Cen*. Unlike *Cen*, *Tfl1* is also expressed in the subapical region of the vegetative shoot meristem. This could account for the early flowering phenotype in *Arabidopsis*, but it would also imply that either *Tfl1* can be induced independently of *Lfy* activity or that *Lfy* possibly has a low activity in vegetative tissue (Ratcliff, 1996).

In Solanaceae

Tobacco *and Petunia* belong to the family of the *Solanaceae*, and have cymose inflorescences. The expression of the *Flo Lfy* homologues *Nicotiana Flo-Like 1* and *2* (*NFL1* and *NFL2*) in tobacco is not restricted to the floral meristem (Kelly *et al.*, 1995). *NFL1* and *NFL2* are strongly expressed in the sepal and petal region of the developing flower but also in the axillary inflorescence meristems. The expression in the axillary meristem might reflect the different inflorescence morphology. However, *NFL1* and *NFL2* transcripts accumulate also in the vegetative apex and in the adaxial surfaces of young leaves (comparable to the expression of *Flo* in bracts). Therefore, the presence of *NFL1* or *NFL2* mRNA cannot serve as a marker for floral commitment in tobacco. nfl Mutants would be very usefull to evaluate the significance of the expression of these genes in vegetative tissue (Kelly *et al.*, 1995).

The *Petunia* mutant *aberrant leaves and flowers* (*alf*) has a normal vegetative development, but its flowers are replaced by cymose inflorescence shoots, which results the formation of highly branched systems (Gerats *et al.*, 1989). The more apical inflorescence shoots of the aging alf mutant can develop sepaloid and carpeloid organs (Tamara Maes, unpublished results). Recently, it has been shown that *Alf* is the *Flo* orthologue of *Petunia* (Ronald Koes and Erik Souer, personal communication).

Intermediaries between meristem and organ identity genes

Are the organ identity genes activated directly by the meristem identity genes, or do they induce downstream genes to perform this function? At least one gene seems to take an intermediary position in *Antirrhinum.*

The *fimbriata* (*fim*) mutants of *Antirrhinum* have variable phenotypes. Weak *fim* mutants develop streaks of green sepaloid tissue on their petals and sometimes have slightly petaloid stamens. The flowers of stronger fim mutants have petaloid sepals in the second whorl, and carpeloid organs in the third whorl, and sometimes sepaloid organs and loss of determinancy can be observed in the fourth whorl (Simon *et al.*, 1994). *Fim* expression is much reduced in *flo* mutants. Strong *flo* mutants are epistatic to *fim* (the double mutant phenotype is identical to the *flo* single mutant) but in combination with a weak *flo* allele, the *fim* phenotype is enhanced, so *Fim* might be induced by *Flo*. In strong *squa* mutants *Fim* expression is delayed but eventually emerges strongly in *squa* floral meristems. The phenotype of *fim* mutants is enhanced in plants that have only one functional *Squa* allele. The double homozygous *squa fim* mutant is more extreme than *squa* alone and exhibits a complete conversion of flowers into inflorescence shoots, which suggests that *Fim* and *Squa* act together in promoting floral meristem identity.

Second whorl sepaloidy and third whorl carpeloidy in *fim* mutants can be interpreted as a weak loss of B function, while the formation of sepals and the loss of determinancy suggests a reduction in C function This corresponds with the observation that *Def* and *Ple* (the B and C function, respectively) expression is delayed and reduced in *fim* mutants.

While *Flo* and *Squa* activities positively regulate the expression of *Fim*, *Fim* induces the floral organ identity genes *Def* and *Ple* in wild type, and thus takes an intermediary position between the meristem and organ identity genes (Simon *et al.*,1994).
The *Fim* homologue *Unidentified Floral Organs* (*UFO)* plays a similar role in mediating between meristem and organ identity genes in *Arabidopsis* (Wilkinson and Haughn, 1995) Mutations in *Fim* and *UFO* seem to decrease the expression of the B function genes in both species However, while the expression of the C function *Ple* is reduced in *Antirrhinum fim* mutants, the expression of *Ag* is not markedly affected in *Arabidopsis ufo* mutant flowers. In addition, *Ag* can be expressed ectopically in the coflorescences of *ufo* mutants, causing them to terminate with a carpel-like structure. So, although there are similarities in the functions of *Fim* in *Antirrhinum* and *UFO* in *Arabidopsis*, there is also a clear functional divergence between the two homologues in the these two species (Ingram *et al.*, 1995).

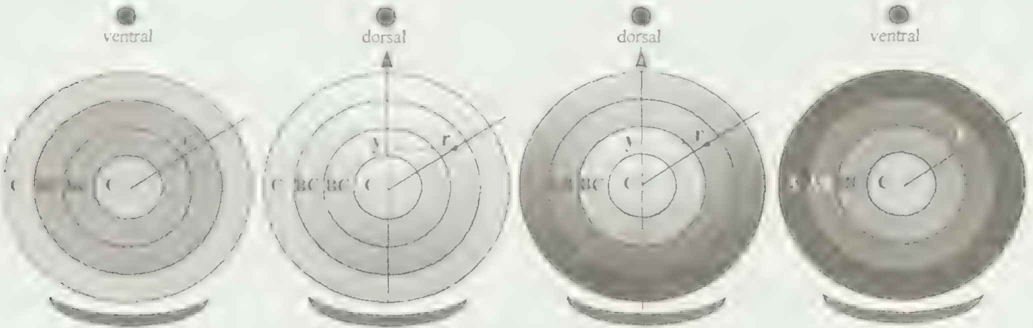
Flower Development and Asymmetry

The development of asymmetry in flowers has been studied on a molecular genetic level mainly in *Antirrhinum majus*.
Antirrhinum flowers are zygomorphic, which means they can be divided in two halves that. mirror image each other (Weberling, 1989) The symmetry plane that bisects the flower is called the dorso-ventral plane, the upper part of the flower is called the dorsal side, the bottom part is called the ventral side The five *Antirrhinum* petals are partially fused to form a tube Based on their shapes and sizes, the petals can be divided into three classes. two big dorsal petals, two intermediate lateral and a small ventral petal. Five stamen primordia are initiated at positions alternating with the petals, the dorsal stamen is aborted and the lateral stamens are smaller than the ventral ones In the fourth whorl, a dorsal and a ventral carpel are united to form the gynoecium (Coen and Nugent, 1994).

In *cycloidea* (*cyc*) mutants, all petals resemble the ventral petals of the wild type flowers, giving the flower a radially symmetrical appearance The abortion of the upper stamen is dependent on CYC activity: in strong *cyc* mutants all five stamens develop to a comparable length (Coen, 1991). The negative effect of CYC activity on the development of the stamen is not third whorl specific, but can be seen as well in mutants that form ectopic anther(oids) like *ovu* and *fis*. In both cases, second whorl organs are (partially) transformed into stamens, and their development is aborted if they occupy dorsal positions (Coen, 1991, Stubbe, 1966).

In *fim* mutants sepaloid streaks of tissue form along the midveins and the margins of the petals. This weak homeotic transformation is most pronounced on the ventral side of the flower (Simon *et al.*, 1994). CYC might also repress the development of the sepal tissue in the second whorl to some extent, a hypothesis which could be verified by analysing the cyc/fis double mutant.

A Polar Coordinate Model has been proposed to explain the combined action of the floral organ identity activities and the asymmetry gene *Cyc*. According to this model, the expression of the organ identity genes changes in function of the distance to the center of the flower while CYC activity varies along the dorso-ventral axis (Coen, 1991, figure 3).



class A/*cyc* double mutant class A mutant wild type *cyc* mutant

Figure 3: The Polar Coordinate Model For Floral Organ Identity and Zygomorphy

The different whorls of the flower are shown as concentric circles. Wild type: the activity of the Organ Identity Functions changes along the radius (r) of the flower, and the asymmetry function Cyc varies along the dorso-ventral axis (y) (III). In cyc mutants, the differential expression along the y-axis is abolished and the flower becomes radially symmetrical (IV). Mutations in organ identity functions result in the homeotic transformations in different whorlsof the flower. For the primordia that will develop into one specific type of organ, the effect of Cyc depends on the position of those primordia a long the axis irrespective of the whorl they occupy. In organ identity and cyc double mutants, the asymmetry of the homeotic mutant dissappears (II, I) sepals ▆, petals ▆, stamens ▒, carpels ☐ . Vertical arrow represents the dorsalising function *Cycloidea* (After Coen, 1991)

The asymmetry of the environment in which the flower primordium normally develops (bract-flower meristem-inflorescence axis) seems to be important for the generation of a dorso-ventral axis in the *Antirrhimum* flower. When the environment is more symmetrical, as is the case for the terminal flower of *cen* mutants, the flower itself doesn't develop zygomorphy, but remains radially symmetrical even if the *Cyc* gene is wild type (Bradley *et al.*, 1996).

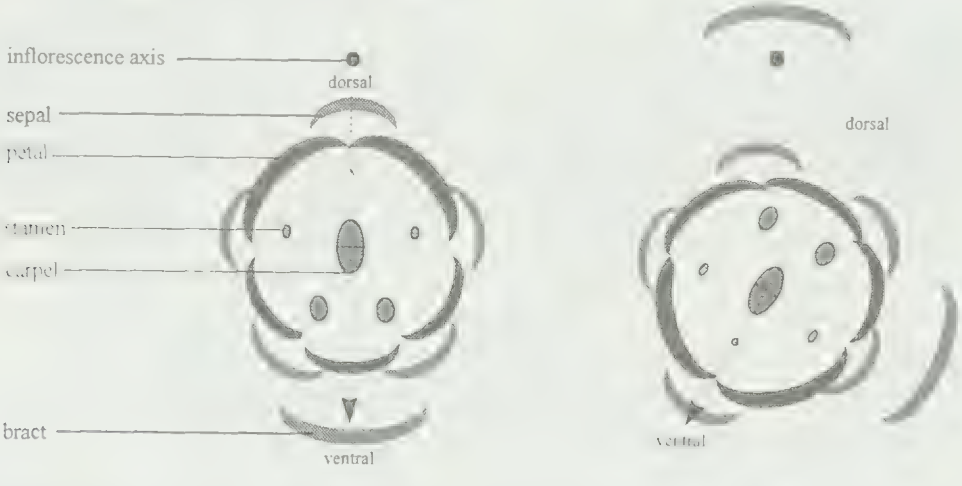
Is there a dorso-ventral inversion in *Petunia* compared to *Antirrhinum*?

Except for *Schizanthus* and *Salpiglossis*, the asymmetry of the flower within the *Solanaceae* is generally a lot less pronounced than in *Antirrhinum*. The dorso-ventral axis of the *solanaceaous* flower is not parallel to the inflorescence axis but rather makes an angle with it (Eichler, 1875).

The ventral side of Petunia hybrida flowers is located distally to the inflorescence meristem. With respect to this orientation, the two ventral fold inward, whereas the three smaller dorsal petals tend to fold backwards. The stamen alternating with the two big petals is clearly less well developed, the lateral stamens are intermediate and the two alternating with the upper petal are largest. In the fourth whorl, the upper and the lower carpel are fused to form the gynoecium. The vigorous growth of the new axillary cymose shoot in the inflorescence pushes the developing flower aside (Turlier and Alabouvette, 1988). The flower ultimately ends up in an orientation *upside down* compared to *Antirrhinum* if one uses petal and stamen size as criteria (figure 4).

Additional support for the idea that a dorso-ventral “inversion” occured between *Antirrhinum* and *Petunia*, comes from the comparison of (weak) homeotic mutants in both species with comparable phenotypes. The *bl* mutant in *Petunia* has a phenotype analogous to *fis* in *Antirrhinum*. Second whorl organs are most affected: on top of a normal tube, antheroids are formed instead of petal lobes. In *Antirrhinum*, the dorsal antheroids are aborted, but not in *Petunia*.

However, in some genetic backgrounds the *bl* phenotype is strongly attenuated, in that case the petal limb relatively well developed, and ectopic patches of antheroid tissue form on petal tissue, mainly on the dorsal side of the flower. Like *fim* mutants in *Antirrhinum*, the greenback mutant of *Petunia* develops sepaloid streaks of tissue mainly along the main veins of the petals. The streaks on dorsal side of the three *Petunia* top petals are wider than those on the ventral side. In all these examples, the homeotic transformations are most pronounced for the three dorsal petals in *Petunia* and the three ventral petals in *Antirrhinum*.



Antirrhinum Petunia

Figure 4: Floral diagrams of *Antirrhinum majus* and *Petunia hybrida*

Direct evidence to prove or disprove the hypothesis of dorso-ventral inversion between these two distantly related members of the *Asterideae* is not available as yet. It would be very useful to obtain *cyc* mutants in *Petunia* hybrida to determine if the slight zygomorphy in *Petunia* is *Cyc* encoded. If so, it may be predicted that the asymmetry in the degree of homeotic transformations in the *gb* and *bl* mutant disappears in a *Petunia cyc* mutant background.

Conclusions and Perspectives

Genes have been isolated that affect the different steps of floral development in different plant model systems. In general, a common mechanism seems to exist for the floral development in different model species. However, it is also clear that considerable functional divergence may arise between different species, even if they are quite close relatives like *Antirrhinum* and *Petunia*.

One question that remains to be answered is how the different inflorescence types arise. In theory, differences in spatial and temporal control of the expression of the two key meristem identity functions *Flo* and *Cen* (or their homologues in other species) could account for the formation of the different inflorescence types that can be observed in nature. The analysis of the molecular basis of those differences could help us to understand the origin of the existing diversity in inflorescence and flower types.

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Signalling in the whole plant during the floral transition

Georges Bernier

Laboratory of Plant Physiology, University of Liège,
Sart Tilman, 4000 Liège, Belgium

Introduction

It is known since long that the floral transition of shoot apical meristems (SAM) is controlled by transmissible signals arising in leaves (Bernier *et al.*, 1981). These signals have been found in most investigated plant species, including plants with the so-called "autonomous" floral induction. The signals include both floral promoters and floral inhibitors.

We have investigated the nature of these signals using several photoperiodic model species that can be induced to flower by a single favourable 24-hour cycle, namely *Sinapis alba*, *Lolium temulentum* Ceres and *Arabidopsis thaliana* Columbia that are induced by exposure to a single long day (LD), and *Xanthium strumarium* that is induced by exposure to a single long night (LN). Note that all species that are induced by a single LD can also be induced by a single displaced short day (DSD), i.e. a SD given at an unusual time within a 24-h cycle. A DSD avoids the lengthening of the light period and thus eliminates the complications related to the extended period of photosynthetic activity characteristic of the LD. All the plant materials were grown, from sowing to end of experiment, in the strictly-controlled conditions of our phytotronic growth rooms.

Most of our work so far has focused on three floral promoters, the carbohydrates, the cytokinins and the polyamines and their interactions.

Carbohydrates

Floral signals from leaf origin are known to move in the phloem sap (Bernier *et al.*, 1981). Since the major component of this sap is sucrose, we have investigated the possible participation of this carbohydrate in the control of the floral transition.

Question 1. Does the level of sucrose in the phloem sap increase as a result of photoperiodic induction of floral initiation? Experimental evidence indicates that in **all** the above species the level of sucrose increases temporarily in the phloem sap of induced plants compared to noninduced plants (Lejeune *et al.*, 1991, 1993, Houssa *et al.*, 1991, Perilleux and Bernier, 1996, Corbesier *et al.*, in preparation). Remarkably, this increase is observed not only in inductive conditions involving a lengthening of the period of photosynthetic CO_2 fixation (exposure to a LD) but also in conditions where induction does not modify (exposure to a DSD) or decreases photosynthetic CO_2 fixation (exposure to a LN).

Question 2: Is the timing of the sucrose increase compatible with the timings of other events of the floral transition, e g. transport of the floral stimulus (as determined by defoliation experiments), SAM activation, initiation of first reproductive (floral) structures? The increase of the sucrose level in the sap occurs very early in all cases and at times quite compatible with the timing of export of the floral stimulus out of induced leaves. In all cases, the sucrose increase precedes clearly the start of SAM activation, as evidenced by the stimulation of cell divisions, and the start of reproductive (floral) morphogenesis. Thus, the transport of more sucrose apparently precedes any increase in the sink activity of SAM. This suggests that the extra-sucrose plays a message-like role.

Question 3: How is the sucrose level changing in the SAM during the floral transition? The sucrose level was found to increase early in the SAM of *S. alba* induced by either one LD or one DSD (Bodson and Outlaw, 1985), supporting the view that supply exceeds demand in this case On the other hand, the sucrose level in SAM stays unaffected in *X. strumarium* induced by one LN (Mirolo, 1988) and *L. temulentum* induced by one LD (King and Evans, 1991), suggesting that here supply and demand are balanced It is striking that a decrease in the sucrose level in the transitional SAM was so far not reported.

Question 4: What is the effect of sucrose on SAM functioning? In *S. alba*, the effect of sucrose in SAM is manifold. It stimulates the energy metabolism, i e. it increases acid invertase activity, mitochondrial number, succinic dehydrogenase activity and ATP production (Bernier, 1988) Increased tricarboxylic acid (TCA) cycle activity in SAM is apparently essential for the floral transition since transgenic potato plants with reduced citrate synthase (a TCA enzyme) activity have a normal vegetative growth but a late-flowering phenotype (Landschütze *et al.*, 1995). In *S. alba*, sucrose also causes other changes, e g it increases the proportion of rapidly-cycling cells (Jacqmard *et al.*, 1996) All these effects of sucrose are events normally seen in the SAM of *S. alba* and other species during the floral transition, but extra-sucrose alone is unable to cause the floral shift in *S. alba* and many other plants.

Question 5: Does the sucrose increase in the sap result from recent photosynthesis or from reserve (starch) mobilization? Merging the data concerning sucrose level in sap with results concerning the supply of recently-synthesized assimilates to the apex in *S. alba* and *X. strumarium* strongly suggests that, when floral induction does not involve a lengthening of the period of photosynthetic CO_2 fixation, a part, at least, of the extra-sucrose comes from starch mobilization In *X. strumarium* exposed to a LN, for example, the increase in sap sucrose occurs at a time when import into the apex of ^{14}C-assimilates (recently-synthesized assimilates) of leaf origin is decreased (Mirolo, 1988; Houssa *et al.*, 1991).

The critical role of starch mobilization in the control of the floral transition is apparent in the starchless *pgm* mutant of *A. thaliana* which has a late-flowering phenotype (Bernier *et al.*, 1993). Interestingly, the *pgm* mutant responds relatively well to one LD but has a very weak flowering response to one DSD (Corbesier *et al.*, in preparation), an induction system in which the plants are presumably relying more heavily on starch mobilization than they do in the LD system.

Interactions between sucrose and cytokinins

Question 6 : Is the extra-sucrose translocated in the phloem sap at floral induction acting only at the SAM? In *S. alba*, experimental evidence shows that the extra-sucrose, besides acting at the SAM, is also acting at the root system where it increases the export of the ribosides of zeatin and isopentenyladenine, two cytokinins, in the xylem sap (Bernier *et al.*, in preparation). This effect of sucrose is essential for the floral shift since (a) when the sucrose supply to the roots is suppressed by bark ringing, flowering is severely inhibited (Bernier *et al.*, 1993), and (b) this inhibition can be entirely relieved by supplying the roots with exogenous sucrose (Bernier *et al.*, in preparation).

Question 7 : Where are the root cytokinins going ? We have obtained evidence that in *S. alba* they move towards the transpiration sites, i.e. essentially the mature leaves (Bernier *et al.*, 1981, P. Lejeune, unpubl. results). Shortly afterwards cytokinins are reexported by these leaves in the phloem sap, but this time exclusively in the form of isopentenyladenine (Lejeune *et al.*, 1994). Several results indicate that the pulse of isopentenyladenine hits the SAM of *S. alba* about 6 hours after the pulse of sucrose (Bernier *et al.*, 1981, 1993). A similar increase in the level of cytokinins in the phloem sap is also recorded in *A. thaliana* induced by a LD (L. Corbesier, unpubl. results) and in *X. strumarium* induced by a LN (Kinet *et al.*, 1994).

Question 8 : What is the effect of the cytokinins on SAM functioning ? In *S. alba*, the effect of a cytokinin applied alone is manifold. It causes an increase of the mitotic and DNA synthetic indices by shortening the durations of the S and G_2 phases of the cell cycle (Bernier *et al.*, 1993 ; Jacqmard *et al.*, 1996). It also causes the breakdown of the vacuoles into smaller vacuolar units (Havelange *et al.*, 1986). All these effects of the cytokinin are events normally seen in the SAM of *S. alba* and other species during the floral transition, but the applied cytokinin is unable to cause the floral shift in *S. alba* and many other plants.

Question 9 : What happens if a combined sucrose + cytokinin treatment is applied to the SAM of *S. alba* ? The combined treatment causes more changes than the addition of the changes produced by the two individual treatments. For example, the duration of the G_1 phase of the cell cycle is reduced only by the combined treatment, not by the individual ones (Bernier *et al.*, 1993 ; Jacqmard *et al.*, 1996). There is thus an interaction between the two signals at the level of the SAM. However, the combined treatment is still unable to cause the floral transition in *S. alba* (Bernier, 1988).

A third signal : putrescine, a compound of the polyamine family

Not all changes of the cell cycle in the SAM, caused by floral induction in *S. alba*, can be mimicked by the combined sucrose + cytokinin treatment (Jacqmard *et al.*, 1996). This suggests that at least another signal is involved in the control of these changes at the floral transition. Published evidence indicates that cytokinins and polyamines are often implicated together in the regulation of the cell cycle (see Dumbroff, 1990). Since polyamines are well known to stimulate or accelerate flower initiation in various plant species (Martin-Tanguy, 1996), a study of their possible participation in the regulation of the floral transition in *S. alba* was desirable.

Question 10 : Does the polyamine level in the phloem sap increase as a result of floral induction? Yes, the level of putrescine (free and conjugated) increases early and markedly in the sap of plants of *S. alba* induced to flower either by a LD or by a DSD (Havelange *et al.*, 1996). Other polyamines are not present in the sap. The extra-putrescine is synthesized in the leaves since there is no increase in the putrescine level of the xylem sap reaching the leaves. The increased export of putrescine from induced leaves occurs at about the same time as the increased export of cytokinins.

Question 11 : What is the effect of putrescine on SAM functioning ? Work to answer this question is still in progress. Application of DFMO, an inhibitor of putrescine biosynthesis, on induced *S. alba* counteracts the effect of floral induction and the DFMO inhibitory effect is completely reversed by application of putrescine (Havelange *et al.*, 1996). DFMO is also preventing the activation of cell cycle activities caused in the SAM by floral induction. Thus, putrescine is a third signal of leaf origin involved in the control of the floral transition of SAM, possibly by participating in the activation of cell cycle activities. Putrescine might play also other functions and further studies are needed to determine exactly its role in this control.

Conclusions

The effect of the photoperiodic induction of flowering on the SAM is mediated by early and dramatic changes in the fluxes of long-distance signals in the entire plant. In *S. alba*, three floral-promoting signals have been identified : sucrose, cytokinins and putrescine. The changes in the fluxes of these signals are well ordered in time and space.

The signalling system in other species exhibit similarities and differences with the system disclosed in *S. alba*. In *A. thaliana*, for instance, sucrose and cytokinins are apparently involved as in *S. alba*, but gibberellins appear as primary floral promoters (Wilson *et al.*, 1992) whereas they do not seem to be implicated in the control of the floral transition in *S. alba* (Bernier *et al.*, 1993).

Signals other than those discussed here remain undoubtedly to be identified, especially the floral-inhibiting signals.

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Signals and their transduction in early plant embryogenesis

Sacco de Vries*, Marcel Toonen, Arjon van Hengel and Andreas Mordhorst.

Agricultural University Wageningen,
Department of Molecular Biology,
Dreijenlaan 3, 6703 HA Wageningen, the Netherlands.
fax +31.8370.83584, email: sacco.devries@mac.mb.wau.nl
* author for correspondence.

Introduction

The plant embryo is characterised by a stereotyped structure thought to be arranged in a number of elements along an apical-basal or longitudinal axis and along a radial axis. From bottom to top the body pattern elements consist of the embryonic root including the root cap and the root meristem, hypocotyl, cotyledons and the shoot apical meristem. In radial fashion, from the outside to the inside, the epidermis, ground tissue and central vascular system are the main tissue types (Jürgens, 1995).

Plant zygotic embryogenesis spans the period of plant development that ranges from the fertilized egg cell, the zygote, to the mature desiccated embryo present in a protective seed. While zygotic embryogenesis, by definition, is dependent on fertilization, the zygote is not the only constituent of the embryo sac or female gametophyte that has this property. Evidence for embryo development *in vivo* without fertilization comes from studies showing so-called apomictic embryos in certain plant species. These apomictic embryos can have a variable origin ranging from the female gametophyte itself and including the unfertilized egg cell (parthenogenesis) to adventitious embryogenesis initiated from the surrounding maternal tissue (Koltunow, 1993). Even cells of the mature plant body, not in direct contact with the female gametophyte can spontaneously form embryos (Taylor, 1967, Yarbrough, 1932).

Under *in vitro* conditions plant embryos can develop from microspores after a variety of inducing treatments depending on the species (androgenesis) (Ferrie *et al.*, 1995) while it is now also possible to produce embryos from *in vitro* fertilized egg cells (Kranz and Dresselhaus, 1996). Finally, tissue cultured cells, first shown in carrot (Reinert, 1959) and later in many different species can be induced to form so-called somatic embryos. An important question concerns the molecular basis of the formation of the single cell destined to produce the embryo.

While the zygote is destined to develop into an embryo and could therefore be defined as an 'embryogenic' cell, it is less clear in other forms of embryogenesis what changes a cell must undergo in order to become an embryogenic cell, capable of forming an embryo. Therefore, in the apparent absence of a single universally applicable signal that renders cells embryogenic, the unravelling of the molecular mechanisms that underlie the process of embryogenic cell formation is a prime area of interest in plant embryogenesis and one that is so far the exclusive domain of the *in vitro* forms of embryogenesis.

In all forms of embryogenesis the same stages are seen as in zygotic embryogenesis Once an embryo is established as such, it appears therefore safe to assume that the mechanisms of pattern formation that lead to the zygotic embryo is used in all other forms of embryogenesis as well The genetic dissection of this process, so far mainly in *Arabidopsis,* maize (*Zea mays*) and in rice (*Oryza sativa*) is therefore likely to yield genes that are also employed under *in vitro* conditions. Advances in particular areas of plant embryogenesis have been reviewed recently (Ferrie *et al.*, 1995; Goldberg *et al.*, 1994; Jürgens, 1995; Kranz and Dresselhaus, 1996; Meinke, 1995; Thorpe, 1995; Zimmerman, 1993).

In this paper the focus will be on three different aspects of plant embryogenesis:
i) the possible correspondence in early events in zygotic and somatic embryogenesis,
ii) the identification of extracellular signal molecules promoting the formation of plant embryos and finally at iii) pattern formation as an intracellular target process of a signal transduction chain.

Correspondence between events in early embryos of different origin

In vitro somatic embryo development was first observed in suspension cultured carrot cells (Reinert, 1959) . In this section, recent work on the induction phase of somatic embryogenesis is discussed in relation to the early phases of zygotic and androgenic embryogenesis in order to compare the various processes with each other. The term 'embryogenic cell' will be restricted to those cells that have completed the transition from a somatic state to one in which no further externally applied stimuli are necessary to produce the somatic embryo (De Jong *et al.*, 1993a). The cells that are in this transitional state and have started to become embryogenic, but still require externally applied stimuli are defined as competent cells (Toonen *et al.*, 1994) After appropriate culture manipulations, usually involving synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D), either alone or in combination with cytokinins, somatic embryos can develop from almost any part of the plant body. However, immature zygotic embryos are a frequently used source of explant material due to the high rate of success in obtaining embryogenic cell cultures.

Several aspects of somatic embryogenesis have been reviewed (De Jong *et al.*, 1993a; Schmidt *et al.*, 1994; Yeung, 1995, Zimmerman, 1993) An important question in this area is whether the first cells that have become competent to form an embryogenic cell in vitro can be recognized and thus identified or isolated. Early work by the group of Komamine suggested that competent cells were 'small, isodiametric and cytoplasmic' (Komamine *et al.*, 1990). In contrast, analysis employing the recording of many thousands of individual carrot suspension cells has shown that competent single cells are of many morphologically different types (Toonen *et al.*, 1994).

An analysis of the events that occur during treatment of hypocotyl explants with 2,4-D has shed light on where the initial population of competent cells originates. Proliferation of provascular cells resulted in a mass of small, rapidly proliferating cells (Guzzo *et al.*, 1994) However, these cells are not yet competent to form embryogenic cells, as demonstrated by cell tracking (Schmidt *et al.*, 1996). Some of the proliferating cells appear to elongate, and a limited number of a particular oval to triangular intermediate cell type has acquired competence to become embryogenic and produce a somatic embryo, again demonstrated by cell tracking (Schmidt *et al.*, 1996).

None of the other cell types, including the small isodiametric rapidly dividing cells released from the explant developed into somatic embryos. So far the study of the transition of somatic cells into competent and embryogenic cells was hampered by the inability to prove that particular early markers for competent and embryogenic cells were indeed absolutely specific for those few cells that are capable of embryogenic cell formation.

Several systems have been described in which it is possible to induce somatic embryos directly from explant cells, without an intervening callus stage. One of the most advanced systems is based on leaf explants of the *Cichorium* hybrid "474" (Dubois *et al.*, 1991). In this system, somatic embryos develop after treatment with growth regulators or by incubation at 35 °C (Decout *et al.*, 1994). The first divisions during embryo development can be synchronised by the addition of glycerol to the incubation medium (Robatche Claive *et al.*, 1992). The nucleus increases in size and is displaced towards the centre of the activated mesophyll cells. The vacuole becomes fragmented and callose is deposited in the cell wall of the embryogenic cell (Blervacq *et al.*, 1995; Dubois *et al.*, 1991). The first division of the embryogenic cell is symmetrical and anticlinal in respect to the orientation of the vascular elements (Blervacq *et al.*, 1995). Subsequent anticlinal divisions lead to the formation of an embryogenic structure that develops into a somatic embryo (Blervacq *et al.*, 1995, Dubois *et al.*, 1991). In other systems somatic embryos develop spontaneously like on megagametophyte tissue of *Pinus taeda* (Becwar *et al.*, 1990) or after thawing of cryopreserved somatic embryos of *Picea sitchensisi* (Kristensen *et al.*, 1994).

Comparing early stages of zygotic and somatic embryo development showed similar developmental patterns in a number of species. In rice, as in most other species, the zygote elongates after fertilization and then divides unequally and transversal whereafter the terminal cell divides in a variable fashion (Jones and Rost, 1989). This is similar to the first divisions of epithelial scutellum cells that develop into somatic embryos (Jones and Rost, 1989). Also somatic embryos of *Vitus* (Altamura *et al.*, 1992) and *Ranunculus* (Konar *et al.*, 1972) follow the same embryonic type of development as their zygotic counterparts. However, early divisions in carrot somatic embryogenesis follow variable division patterns resembling e.g. the Crucifer type (McWilliam *et al.*, 1974) but also symmetrical divisions have been observed (Toonen *et al.*, 1994). Similar results were obtained during somatic embryogenesis in alfalfa (*Medicago sativa*) where initial divisions were less precise when compared to the zygotic divisions (Dos Santos *et al.*, 1983). Somatic embryos often lack a suspensor (Toonen *et al.*, 1994; Xu and Bewley, 1992) and can develop via morphologically distinct cell clusters (Toonen *et al.*, 1994) showing that somatic embryo development may be more flexible than the zygotic development.

Expression patterns of embryogenesis related mRNAs (Franz *et al.*, 1989; Sterk *et al.*, 1991; Wurtele *et al.*, 1993) and the embryogenic ECP40 protein (Kiyosue *et al.*, 1993) are similar during early somatic and zygotic embryo development. However, later in development expression patterns of storage proteins of somatic embryos of alfalfa deviate from the zygotic ones. The transcription of storage protein mRNA is comparable in zygotic and somatic embryos but due to physiological conditions protein translation is altered in somatic embryos (Krochko *et al.*, 1994; Pramanik *et al.*, 1992).

Another example of the close correspondence between somatic and zygotic embryogenesis comes from the analysis of markers for suspension cells that are in the process of acquiring embryogenic competence. The formation of cells that have acquired the ability to become embryogenic remains an area that is difficult to address experimentally. The reasons for this are that the number of cells that complete the transition between somatic cell and somatic embryo are few and ill-defined. For example, it has been proposed that carrot suspension cells decorated with the JIM8 AGP cell wall epitope are in a transition between competent and embryogenic cell state (Pennell *et al.*, 1992). This was based on the labelling of a subpopulation of single cells with the JIM8 antibody and also on the occurrence of labelled cells in embryogenic cultures only.

Cell tracking of JIM8-labelled cell populations however failed to demonstrate a causal relationship between JIM8 labelling and embryo formation (Toonen *et al.*, 1996). Given the demonstrated promotive effects of AGPs, it is possible that the JIM8 decorated cells perform some accessory function in embryogenesis. These results prompted a search for more reliable markers. Screening of cDNA libraries yielded clones of which the expression pattern was correlated with embryogenic potential. One of these genes encoded a leucin-rich repeat receptor-like kinase designated Somatic Embryogenesis Receptor Kinase (SERK). Employing cell tracking, the very first cells competent to form somatic embryos that appear on a 2,4D-treated carrot explant were identified and shown to express the SERK gene. In embryogenic suspension cultures, less then 1 % of the cells expressed SERK, corresponding to the number of competent cells. Definite proof for the SERK gene as marker has now been obtained from promoter-luciferase studies in conjunction with cell tracking (Schmidt *et al.*, 1996). During somatic embryogenesis, SERK-expression was transient and disappeared at the early globular stage. During zygotic embryogenesis, SERK-expression was only detectable by RT-PCR after pollination and again transiently in young zygotic embryos of up to 100 cells. No expression was seen in any other phase of the plant life-cycle. These results demonstrate that competent cell formation in vitro shares a highly specific signal transduction chain with early zygotic embryogenesis, after but not before fertilization (Schmidt *et al.*, 1996). The nature of the SERK-activating signal is at present unknown. We hope that SERK and other markers will aid in understanding the formation of cells competent to form embryos in vitro.

Extracellular signal molecules

A point of discussion that has surfaced in the field of somatic embryogenesis is whether the cells that are in the transition between the somatic state and the embryogenic state progress in a cell-autonomous way under the influence of exogenous growth regulators or whether they receive specific signals from neighbouring cells. Evidence for the importance of cell to cell communication in embryogenic cell formation comes from the beneficial effect of e.g. suspension cell cultures (Kranz *et al.*, 1991) or embryogenic microspore cultures (Holm *et al.*, 1994) on *in vitro* zygote culture. Similar effects have been observed in somatic embryogenesis by addition of conditioned medium (De Vries *et al.*, 1988). Whether this points to the existence of classes of specific molecules or whether there is a more general effect on cell division, as demonstrated in single cell cultures (Spangenberg *et al.*, 1985) is not known yet.

The observation that the carrot somatic embryo mutant ts11, arrested in the globular stage, can be rescued by addition to the medium of a secreted endochitinase (De Jong *et al.*, 1992, De Jong *et al.*, 1995) revealed an unexpected function of this class of plant hydrolytic enzymes in somatic embryo development. The arrested phenotype of ts11 is the result of a transient decrease in the amount of the EP3 chitinase present (De Jong *et al.*, 1995). The EP3 chitinase is a class IV endochitinase and is one of the five members of a gene family (Kragh *et al.*, 1996). Two of the EP3 chitinases were purified and found to be active in ts11 rescue, as was a related class I chitinase. The EP3 genes are expressed in a certain subset of suspension cells in both wild-type and ts11 suspension cultures, but not in somatic embryos themselves (Van Hengel and De Vries, to be published). This suggests a 'cross-feeding' mechanism of action by which secreted proteins produced by one cell type influence the development of embryos from another cell type, and pointing to the existence of cell-to-cell communication events involved in somatic embryogenesis.

Another example of such communication in cell culture may be found in certain arabinogalactan proteins (AGPs). Recently it has been shown that AGPs can promote embryogenic cell formation in suspension cultures of carrot (Kreuger and Van Holst, 1995, Kreuger and Van Holst, 1993) and *Pinus* (Egertsdotter and Von Arnold, 1995). AGPs are proteoglycans with poly- and oligosaccharide units covalently attached to a central protein core. AGPs react with the ß-glycosyl Yariv reagent (Fincher *et al.*, 1983; Kreuger and Van Holst, 1996). Binding of ß-D glycosyl Yariv reagent to cell wall AGPs of rose suspension cells inhibited growth in a reversible fashion, probably due to suppression of the cell cycle eventually in combination with prevention of cell expansion (Serpe and Nothnagel, 1994).

In *Arabidopsis* seedlings and in carrot suspension cultures a similar effect of ß-D glycosyl, but not of AGP-unreactive α-galactosyl Yariv reagents was observed (Willats and Knox, 1996). Such a role for AGPs seems in line with their expression *in planta*, where e.g. JIM4 reactive AGP epitopes mark emerging anatomical patterns in developing carrot somatic embryos (Stacey *et al.*, 1990). Based upon expression of JIM4 and JIM13 epitopes in the carrot root apex a function for AGPs in determination of the cell fate has been postulated (Knox *et al.*, 1991, Knox *et al.*, 1989). Maize coleoptiles that are committed to undergo programmed cell death acquire a specific set of AGP epitopes (Schindler *et al.*, 1995). These examples suggest that AGPs may play a role in determination of cell fate and cell differentiation (Chasan, 1994; Knox, 1995).

If components of the conditioned medium of plant cell cultures such as chitinases and AGPs have a beneficial effect on several stages of somatic embryo formation, it is of interest to determine where such molecules are found during zygotic embryogenesis. Indeed, developing seeds have shown to be a rich source of AGPs able to promote embryogenic cell formation in tissue culture (Kreuger and Van Holst, 1993), while the carrot chitinase EP3 genes appear to be expressed in the integuments and in the endosperm between 3 and 14 DAP (Van Hengel and De Vries, to be published). This seems to point to a quite intimate role between the embryo and the surrounding sporophytic and gametophytic tissues, a topic that can now also be addressed through *Arabidopsis* mutants that show endosperm development without fertilization (Ohad *et al.*, 1996). The existence of these *fertilization independent endosperm* (*fie*) mutants suggest that endosperm and embryo development are genetically divergent

The *fie* mutation also has a female gametophytic embryo lethal phenotype, and while the reasons for this are unclear, it may support the notion that early embryogenesis is dependent on endosperm formation This idea has previously been exploited through the use of coconut milk in plant tissue culture experiments and which eventually led to the identification of plant cytokinins. While it is clear that much further work is necessary to prove the existence of a signal transduction system that has as its target the early embryo or embryo intials in vitro and the zygote or early embryo in ovulo, many different results appear to point in this direction A more complete discription of these examples is to be found in Mordhorst *et al.* (1997).

Pattern formation as a target process for extracellular signal molecules

During *Arabidopsis* embryogenesis, the zygote divides asymmetrically in the future apical-basal axis, however, a radial axis is initiated only within the eight-celled embryo Mutations in the GNOM, KNOLLE, and KEULE genes affect these processes. *gnom* zygotes tend to divide symmetrically; *knolle* embryos lack oriented cell divisions that initiate protoderm formation; and in *keule* embryos, an outer cell layer is present that consists of abnormally enlarged cells from early development Pattern formation along the two axes is reflected by the position-specific expression of the *Arabidopsis* lipid transfer protein (AtLTP1) gene In wild-type embryos, the AtLTP1 gene is expressed in the protoderm and initially in all protodermal cells; later, AtLTP1 expression is confined to the cotyledons and the upper end of the hypocotyl Analysis of AtLTP1 expression in *gnom*, *knolle*, and *keule* embryos showed that *gnom* embryos can also have no or reversed apical-basal polarity, whereas radial polarity is unaffected *knolle* embryos initially lack but eventually form a radial pattern, and *keule* embryos are affected in protoderm cell morphology rather than in the establishment of the radial pattern (Vroemen *et al.*, 1996, and references cited therein).

While the cellular origin of somatic embryos is quite different from zygotic ones, both the radial and the apical-basal pattern formation takes place in correct fashion. Whether apical-basal pattern formation in somatic embryos depends on an axis of polarity in competent single cells comparable to the axis of polarity in the zygote is not clear. While asymmetric cell division has often been reported to accompany embryogenic cell formation (reviewed in De Jong *et al.*, 1993a), at least in established carrot cultures such a correlation was not evident (Toonen *et al.*, 1994). However, in the absence of experimental data on the nature of the mechanisms that set up and fix polarity in the zygote or in single competent cells, it remains to be established whether apical-basal axis formation in plant embryos always requires such polar cells or whether alternative mechanisms exist.

The radial pattern in somatic embryos most likely proceeds by specification of inside and outside cells, as it occurs in zygotic embryos. In this respect it is of interest to note that at least one marker of embryogenic cells in culture systems encodes a protein associated with epidermal cells (Sterk *et al.*, 1991). However, as in zygotic embryogenesis (Vroemen *et al.*, 1996), the molecular mechanisms by which the radial pattern in somatic embryos is established are unknown.

In several systems the question whether auxin gradients are instrumental in apical-basal pattern formation have been addressed. Inhibition of polar auxin transport by application of 2,3,5-triiodobenzoic acid (TIBA) or 9-hydroxyfluorene-9-carboxylic acid (HFCA) in *in vitro* cultured zygotic globular embryos of Indian mustard (*Brassica juncea*) led to the formation of fused cotyledons. The treated embryos did however form shoot and root meristems (Liu *et al.*, 1993), suggesting that apical-basal pattern was complete before the globular stage, or is not dependent on polar auxin transport. Globular and oblong carrot somatic embryos treated with *N*-(-1naphthyl)-phthalmic acid (NPA) or TIBA did not develop roots or shoots, but only increased in size (Schiavone and Cooke, 1987) suggesting that in somatic embryos other mechanisms of apical-basal pattern formation operate or that the time at which this is established is later than in zygotic embryos. In *in vitro* cultured globular wheat embryos addition of TIBA influenced the position and development of the shoot apical meristem, while no root meristem was formed (Fisher and Neuhaus, 1996).

Dramatic effects of exogenous auxin were also reported, perhaps through disturbance of the endogenous auxin gradients in the globular embryo (Cooke *et al.*, 1993, Michalczuk *et al.*, 1992). While suggestive of an important role of polar auxin transport in some aspects of pattern formation, it is not clear at all whether the proposed auxin gradients necessary are indeed established already in the pre-globular embryo. While intracellular levels of the synthetic auxin 2,4-D and the endogenous indole-3-acetic acid (IAA) have been measured on whole clusters and embryos (Ivanova *et al.*, 1994; Michalczuk *et al.*, 1992) no auxin gradients have been demonstrated so far.

The establishment of somatic embryogenesis in *Arabidopsis* allows to combine the molecular genetic approaches used in zygotic embryos (Mayer *et al.*, 1991, Meinke, 1995) with cellular (Van den Berg *et al.*, 1995) and biochemical approaches (De Jong *et al.*, 1992; De Jong *et al.*, 1993b). While immediately useful for e.g. mutant embryo rescue and phenotypical analysis, other advantages are the availability of sufficient quantities of developing staged embryos and eventually the establishment of screens for mutants with an altered ability to form somatic embryos. Embryogenic callus cultures of *Arabidopsis* have been obtained by culturing immature zygotic embryos of the ecotype Columbia (Pillon *et al.*, 1996).

Cell lines with even higher embryogenic capacity were obtained from a mutant, *primordia timing 1* (*pt-1*), allelic to *altered meristem program-1*, characterised by a higher than normal ability to regenerate via organogenesis (Chaudhury *et al.*, 1993). The embryogenic capacity of these cell cultures could be maintained up to now for more than one year. Somatic embryos were initiated in the presence of 2,4-D and developed further on hormone free medium. Plants derived from somatic embryos were grown to full maturity in the greenhouse. While all apical-basal and radial embryo pattern elements, as seen in the zygotic embryo, are present in somatic embryos as well, differences concerning the number of cell layers and the number of cells per cell layer between *Arabidopsis* zygotic and somatic embryos were found.

This finding demonstrates the flexibility of the developmental processes taking place during plant embryogenesis and underscores the separation of body pattern formation and cell division patterns (Mordhorst and De Vries, to be published).

Concluding remarks

Several parallel developments in the field of plant embryogenesis have occurred that should predict a rapid progress in several areas. The genetic analysis of mutant embryos, sofar mainly in *Arabidopsis*, but also available in rice and maize, characterized by abberations in their morphology has yielded several genes instrumental in early embryogenesis. While it is not yet clear how the identity of the genes obtained translates into the mutant morphology observed, it is clear that cytokinesis is an important component of the making of the plant embryo (Lukowitz *et al.*, 1996). In vitro systems have been instrumental in the identification of novel signal molecules such as AGPs and chitinases or products of their enzymatic activity as compounds able to promote embryo development.

The availability of cell tracking to determine effects on individual cells in the midst of hundreds or thousands of non-responsive cells will be invaluable to unravel the possible connection between externally applied signal molecules and the resulting developmental changes. Classical growth regulators such as auxins are reported to be able to mimick the phenotypes of certain embryo pattern mutants. Eventually, we hope that the availability of somatic embryogenesis in *Arabidopsis* may help to elucidate the connection that may exist between both classical and novel plant growth regulators in the formation of pattern in the early embryo. Clearly, one of the challenges of the future will be to combine and integrate the various approaches possible, such as genetic analysis aimed at embryo cell specification and pattern formation, reverse genetics and targetted gene disruption of embryo-expressed genes with a biochemical analysis of signalling molecules affecting plant embryogenesis. Such a possibility now exists in the form of the European Plant Embryogenesis Network (EPEN) coordinated by S. de Vries, WAU and funded by the European Commission as part of the Biotechnology Program. This network comprises 53 laboratories each studying different forms and aspects of plant embryogenesis.

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Molecular aspects of somatic embryogenesis in conifers

Chandelier A.[1,2*], du Jardin P. [1#], Paques M.[2]

# : corresponding author
1. : Département de Biochimie et Biologie Appliquées, Unité de Biologie Végétale, Faculté Universitaire des Sciences Agronomiques de Gembloux, 2 Passage des Déportés, B-5030 Gembloux, Belgique.
2. : AFOCEL, Station de Biotechnologies, Domaine de l'Etançon, F-77370 Nangis, France.
* present address : Département de Biochimie et Biologie Appliquées, Unité de Phytopathologie, Faculté Universitaire des Sciences Agronomiques de Gembloux, 2 Passage des Déportés, B-5030 Gembloux, Belgique.

1. Summary

Somatic embryogenesis provides a means to propagate high-value conifers of several species. However, a better knowledge of the molecular events which control this process is essential to overcome some problems such as the variable embryogenic potential of both the explant and the genotype, problems which limit the development of somatic embryogenesis at an industrial scale (Paques *et al.*, 1995). Moreover it is important to evaluate the clonal fidelty of the plantlets regenerated via somatic embryogenesis. A brief review of research carried out on these subjects is described, as well as original data obtained in our laboratories.

2. Introduction

Somatic embryogenesis, an *in vitro* process where embryos arise from somatic cells, was first described in conifers by Hakman who obtained embryogenic cells from immature zygotic embryos of *Picea abies* (L.) Karst. (Hakman *et al.*, 1985). Later on, this process has been extended to other conifers, and to more mature tissues (for a review, Tautorus *et al.*, 1991; Dunstan *et al.*, 1995).

Different stages of development are commonly described in conifer somatic embryogenesis (Hakman and von Arnold, 1985). Briefly, in *Picea abies* (Bercetche, 1989; Ruaud *et al.*, 1992; Ruaud *et al.*, 1993), on an initiation medium containing auxin and cytokinin, cotyledons and young needles develop an undifferentiated callus called "non embryogenic " (NEC) and white, translucent and mucilaginous masses of cells which constitute "embryonal suspensor masses" (ESMs). These ESMs, which will give rise to cotyledonary somatic embryos, are composed of two cell types (Hakman *et al.*, 1985): small cytoplasmic cells forming the embryonal head supported by long vacuolated cells which constitute the suspensor.

In contrast, NEC do not show any anatomical organization. When they are sufficiently developed, ESMs are separated from the NEC and subcultured on a multiplication medium. The conversion of ESMs in mature cotyledonary somatic embryos is promoted on a maturation medium containing abscisic acid and a suitable carbohydrate source. Finally, plants (also called emblings) are regenerated from somatic embryos by transfer on a germination medium devoid of phytohormones.

Somatic embryogenesis is a promising method for conifer propagation. Indeed, it offers the possibility to produce a huge number of propagules of high-value genotypes. It is also a source of totipotent material for cryopreservation (Kristensen *et al.*, 1994; Bercetche *et al*, 1990) and transformation (Charest *et al.*, 1996; Bercetche *et al*, 1993). Moreover, from a fundamental point of view, somatic embryogenesis constitutes an experimental system for studying molecular events which are associated with the development of zygotic embryos. However, several problems still limit the development of this technique at an industrial scale. In this context, molecular biology appeared as an interesting tool for the understanding (and maybe the control) of the mechanisms underlying somatic embryo development and the analysis of clonal fidelity of regenerated plants.

3. Molecular events in conifer somatic embryogenesis

3.1. Characterization of the embryogenic state: comparison between embryogenic and non embryogenic cultures

In angiosperms, and mainly in carrot, research indicated that extracellular proteins secreted in the culture medium conditioned the somatic embryogenesis process (van Engelen and De Vries, 1992). In contrast, in conifers, only a few studies report on this subject. Comparisons between embryonal suspensor masses and non embryogenic calli have been carried out mainly at the protein level for understanding the events which are associated with the development of somatic embryos in conifers. Domon and coworkers (1994) identified different electrophoretic patterns of extracellular proteins between ESMs and NEC in *Pinus caribaea* var. *hondurensis.*

In contrast, very similar patterns were shown within each cell culture type. Amino acid sequence homologies were found between GP111, one of the polypeptides representative of the embryogenic lines and germin-like proteins, markers of embryo development in cereals (Domon *et al.*, 1995). In *Picea abies*, Chauveau *et al.* (1994), identified two polypeptides of 35 kDa and 63 kDa, specific to NEC and ESMs respectively. Further analysis demonstrated that the 63 kDa polypeptide was extracellular and glycosylated. Moreover, the detection of this polypeptide was proven to be associated with the appearance of the ESMs on the explant, suggesting that it could be used as an early marker of somatic embryogenesis in this species.

While the efficiency to produce ESMs is influenced by the explant reactivity (juvenile explants are more receptive), some individuals called "recalcitrant genotypes" are not able to differentiate ESMs on an initiation medium from any explant type. This selection of "competent genotypes" (i.e. genotypes able to differentiate somatic embryos on an initiation medium) could have a serious impact on the genetic diversity of forest trees, if used for amplifying populations. Moreover, the impossibility to initiate a somatic embryogenesis process from explant of mature trees having well-established phenotypic characteristics limits the cloning of elite trees. Departing from the results described above where ESMs and NEC displayed distinct extracellular protein contents, some researchers were interested by the possibility to convert NEC from either recalcitrant genotypes or non reactive explant in ESMs, by cocultivating the two types of cell cultures ("nurse cultures"). Hence, in *Picea abies*, Westcott (1993) succeeded in converting NEC initiated from explant of a 26-years-old trees into ESMs.

Experiments carried out in our laboratories on *P. abies* aimed at comparing ESMs and NEC mitochondrial DNA structure (mtDNA). Indeed, RFLP analysis carried out in several angiosperms (Shirzadegan *et al.*, 1991, Rode *et al.*, 1987) and also in *Larix* (De Verno *et al.*, 1994) demonstrated the instability of this genome during *in vitro* culture. A mitochondrial library was constructed in plasmid vectors and mitochondrial clones (about 200) were used as RFLP probes for comparing the mtDNA structural organi-zation in ESMs and NEC initiated from young cotyledons for 6 genotypes and separately multiplied for over two years. Two probes identified qualitative polymorphisms between ESMs and NEC from the same genotype (Chandelier *et al.*, in preparation). Moreover, one of the two probes displayed homology with extrachromosomal DNA elements identified in the *P. abies* mitochondrion. Sequencing of the two polymorphic probes failed to provide any coding function.

3.2. Characterization of the maturation phase

The conversion of ESMs into mature somatic embryos is conditioned by exogenously supplied substances. Hence, Misra *et al.* (1993) on comparing gene expression during the embryogenesis process of both zygotic and somatic embryos, found that the presence in the maturation medium of abscisic acid (ABA) and osmoticum was essential for promoting the expression of storage proteins (markers of embryo maturation) similar to those observed in zygotic embryos. More recently, Dong and Dunstan (1996) identified, by differential screening, cDNA clones associated with somatic embryo development in *Picea glauca* [Moens] Voss. Three major sets of genes were categorized according to the developmental stage of the embryo. Predicted amino acid sequence analysis revealed homology with different proteins like late-embryogenesis-abundant proteins (LEA), storage proteins or heat-shock proteins for some of them, while others failed to provide any sequence homology with known sequences.

In *Picea abies*, Egersdotter *et al.* (1993) demonstrated the existence of two types of embryogenic cell lines which were called A and B. The type A cells were able to develop mature somatic embryos on a maturation medium while type B did not. However, by providing secreted proteins from type A to type B cells, the latter were able to produce cotyledonary embryos on a maturation medium, suggesting that extracellular proteins are essential for promoting the maturation of somatic embryos.

3.3. Upon regeneration : analysis of possible somaclonal variation

A possible problem encountered when using *in vitro* techniques is somaclonal variation, i.e. genetic changes in plants regenerated from tissue culture (Evans, 1989).

Regarding the long development cycle of trees, it is important to have "tools" which enable to rapidly check if regenerated plants are "true-to-type" relative to their respective mother plants. Different cellular and molecular biology techniques have been used in order to characterize the potential stability of conifers during *in vitro* culture. Hence, in *P. abies*, Mo *et al.* (1989) showed that nuclei isolated from ESMs initiated both from mature zygotic embryos and regene-rated plants retained the same DNA content (2n = 24) as seedlings.

In contrast, Fouré (1995) detected genomic mutations (i.e. monoploïdy, trisomy, tetraploïdy) by chromosome counting in five years-old ESMs from the same species. However, most of these mutated ESMs were not able to develop into mature embryos. In *P. mariana*, Isabel *et al.* (1993) did not detect any genetic variability between ESMs and their corresponding somatic embryos by RAPD analysis with 10 primers.

In our laboratories, we compared the mtDNA organization in plants regenerated from somatic embryos and their corresponding mother plants in *P. abies*. By initiating the somatic embryogenesis process from cotyledons rather than mature or immature zygotic embryos, the mother plant was preserved (Chaveau *et al.*, 1995) and used for the detection of possible rearrangements in the mtDNA of emblings. As there is a great genetic heterogeneity between individuals in forest trees, the survival of the mother plant was thus an important advantage. The mtDNA organization of plants regenerated from somatic embryos after 37, 100 and 200 subcultures of ESMs was compared to that of their respective mother plants at sequences homologous to mtDNA regions which previously detected RFLPs between ESMs and NEC (*cfr* point 3.1). While qualitative polymorphisms were detected between ESMs and their respective mother plants, no variation was detected in plants regenerated from ESMs of 37 and 100 subcultures, suggesting that the structural modifications observed in ESMs were transient.

The reversibility of mtDNA structural changes affecting ESMs has already been observed in wheat (Hartmann *et al.*, 1987). In contrast, RFLPs were detected in 8 plantlets (out of 8 analysed) and corresponding to plants regenerated from ESMs of 200 subcultures (about 4 years in culture) The experiment, carried out for 2 other genotypes on 8 plantlets (out of 8 analysed) revealed that plants regenerated after 80 subcultures of ESMs did not recover the mtDNA organization of their respective mother plants Despite that fact, no phenotypic variation has been observed either in *in vitro* or *ex vitro* conditions. It is the first time that qualitative mitochondrial DNA structural changes are described in emblings. These mitochondrial DNA rearrangements need to be considered according to the growth pattern of those somatic plants recorded by AFOCEL in field trials.

4. Conclusion

Since the experiments of Hakman *et al.* in 1985 on *Picea abies*, numerous experiments have been carried out on somatic embryogenesis in conifers. This *in vitro* technique enabling the mass propagation of high value clones has great potential in forest tree breeding programs. However, the understanding of the molecular events which control the differentiation of somatic embryos in conifers is still in its infancy.

When comparing the few experiments carried out on this subject in conifers with those carried out in angiosperms, and mainly in carrot, it appears that the molecular events which characterize the embryogenesis process in the two plant groups are often similar Indeed, while experiments with carrot revealed that extracellular proteins secreted in the culture medium were involved in the embryogenesis process (for a review, Zimmerman, 1993, van Engelen and De Vries, 1992), works characterizing the embryogenic state in conifers demonstrated variable extracellular proteins in ESMs and NEC. Moreover, recent experiments showed that, like in angiosperms, genes specific to conifer somatic embryo development were differentially expressed according to the differentiation state of embryos.

Finally, proteins involved in angiosperm embryogenesis (for instance the LEA proteins or germins) are also involved in conifer embryogenesis. This suggests that, in some cases, the carrot model system can also be used for characterizing embryogenesis in conifers.

While quantitative changes in the relative abundance of some mtDNA restriction fragments were observed between ESMs and NEC in *Larix* (De Verno *et al.*, 1994), qualitative mtDNA structural changes were observed in *Picea abies* somatic cell cultures (ESMs *vs* NEC) (Chandelier *et al.*, in preparation). The two mitochondrial DNA regions involved in these rearrangements were sequenced but failed to provide any known coding function. Moreover, plants regenerated from ESMs of different subcultures, and genotypes were compared to their respective mother plant at sequences homologous to the two mtDNA regions unstable *in vitro*. In some cases, regenerated plants did not recover the mtDNA organization of their mother plants. The possible phenotypic significance of these mtDNA rearrangements is under investigation by AFOCEL.

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Aspects of morphogenesis of leaves, flowers and somatic embryos

Lectures given during a joint meeting of the *Belgian Plant and Tissue Culture Group* BPTCG and the *F.W.O. Research Network Plant Morphogenesis* held in the Arenberg Castle, K.U.Leuven, December 6, 1996.
Contributions include aspects of plant modelling, leaf and flower development and somatic embryogenesis, highlighting mathematical, biophysical, physiological, genetic and molecular approaches.
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